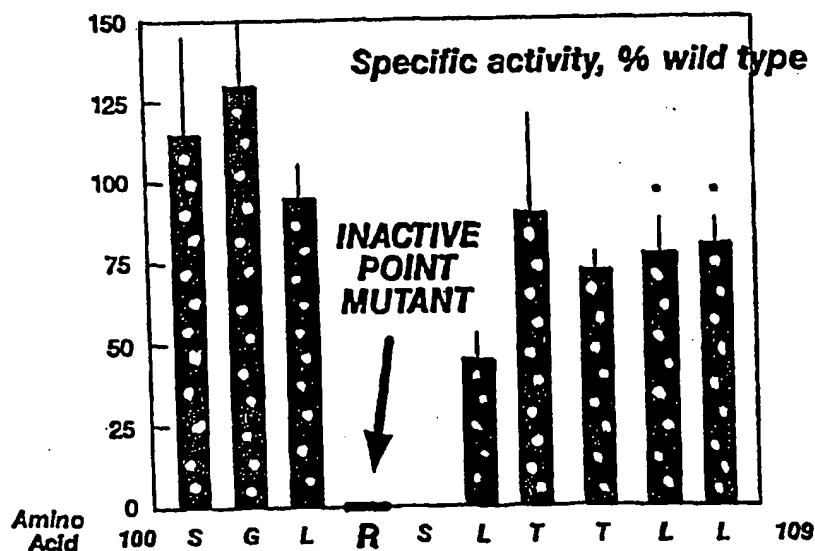




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(54) Title: **ERYTHROPOIETIN WITH ALTERED BIOLOGICAL ACTIVITY****ARGININE 103 IS ESSENTIAL FOR EPO'S ACTIVITY**

(57) Abstract

The invention relates to DNA encoding modified, secretable erythropoietin proteins whose ability to regulate the growth and differentiation of red blood cell progenitors are different from the wildtype recombinant erythropoietin. The invention also relates to methods of modifying or altering the regulating activity of the secretable erythropoietin proteins and the use of the modified secretable erythropoietin proteins, for example, in *in vivo* therapeutics.

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ERYTHROPOIETIN WITH ALTERED BIOLOGICAL ACTIVITY

RELATED APPLICATIONS

This application claims priority to U.S. Serial No. 09/017,631, filed on February 3, 1998, which is a
5 continuation-in-part application of U.S. Serial No. 08/808,881 which was filed on February 28, 1997 which is a divisional of U.S. Serial No. 08/383,743 filed February 2, 1995 issued as U.S. Patent No. 5,614,184 on March 25, 1997, which is a continuation-in-part
10 application of U.S. Serial No. 08/113,080, filed August 26, 1993, now abandoned, which is a continuation-in-part application of U.S. Serial No. 07/920,810, filed July 28, 1992, now abandoned. The teachings of these related applications are incorporated herein by reference.

15 BACKGROUND OF THE INVENTION

The glycoprotein hormone erythropoietin regulates the growth and differentiation of red blood cell (erythrocyte) progenitors. The hormone is produced in the fetal liver and adult kidney. Erythropoietin
20 induces proliferation and differentiation of red blood cell progenitors through interaction with receptors on the surface of erythroid precursor cells.

Several approaches have been employed to identify those features of the protein that are relevant to its
25 structure and function. Examination of the homologies among the amino acid sequences of erythropoietin

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proteins of various species has demonstrated several highly conserved regions (McDonald, J.D., et al., *Mol. Cell. Biol.* 6: 842-848 (1986)).

Oligonucleotide-directed mutagenesis has been used
5 to prepare structural mutants of erythropoietin, lacking specific sites for glycosylation. Studies indicate that N-linked carbohydrates are important for proper biosynthesis and/or secretion of erythropoietin. These studies also show that glycosylation is important for in
10 vivo, but not in vitro, biological activity. (Dube, S., et al., *J. Biol. Chem.* 263:17516-17521 (1988); Yamaguchi, K., et al., *J. Biol. Chem.* 266:20434-20439 (1991); Higuchi, M., et al., *J. Biol. Chem.* 267:7703-7709 (1992)).

15 Studies with monoclonal anti-peptide antibodies have shown that the amino terminus and the carboxy-terminal region (amino acids 152-166) of erythropoietin may be involved with biological activity. It has also been demonstrated that antibodies to amino acids 99-119
20 and 111-129 block the hormone's biological activity, apparently by binding to two distinct non-overlapping domains (99-110 and 120-129). (Sytkowski, A.J. and Donahue, K. A., *J. Biol. Chem.* 262:1161-1165 (1987)). Thus, it was hypothesized that amino acids 99-129 were
25 important in the formation of a functional region involved in receptor recognition, either through forming a necessary component of the protein's tertiary structure or through direct participation in receptor binding, or both.

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Preliminary experiments suggested that alterations in localized secondary structure within the 99-129 region resulted in inactivation of erythropoietin. Therefore, a possible structural role for amino acids 5 99-129 has been postulated. Recently, a series of experiments indicated that amino acids 99-110 (Domain 1) play a critical role in establishing the biologically active conformation of human erythropoietin. (Chern, Y., et al., *Eur. J. Biochem.* 202:225-229 (1991)).

10 These Domain 1 mutants, in which a group of three amino acids was deleted and replaced by two different amino acids, were found to be biologically inactive. Furthermore, these mutations in Domain 1 inhibited the secretion of the mutant erythropoietin into cell culture 15 medium. (Chern, Y., et al., *Eur. J. Biochem.* 202:225-229 (1991)). Inhibition of secretion in mammalian cells is consistent with a profound structural change of the polypeptide hormone. Profound structural changes could significantly affect the ability of the hormone to 20 interact with its cognate receptor. Thus, these mutant erythropoietin polypeptides are not suitable for elucidating the structure/function relationship that exists between erythropoietin and its cellular receptor. Nor are these mutants suitable erythropoietin 25 antagonists for use, for example, in therapeutic treatment of polycythemia, or over production of erythropoietin. Thus, it would be beneficial to precisely determine which amino acids are critical to the erythropoietin polypeptide to maintain a stable, 30 biologically active conformation which retains its

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secretable properties and its ability to bind to the erythropoietin receptor.

Moreover, the precise determination of critical amino acid residues would be useful to alter the biological activity of erythropoietin, either decreasing or increasing one or more biological properties of the protein.

SUMMARY OF THE INVENTION

The present invention relates to isolated DNA encoding mutated erythropoietin proteins which have altered biological activity, yet retain their secretable properties (i.e., secretable erythropoietin proteins).

In one embodiment, the present invention relates to isolated DNA encoding secretable erythropoietin proteins which have at least one amino acid residue in Domain 1 which differs from the amino acid residue present in the corresponding position of wildtype erythropoietin and which have altered ability to regulate the growth and differentiation of red blood cell progenitors. Domain 1 of the mutants described herein refers to the amino acids which correspond to amino acids 99-110 (SEQ ID NO: 1) of the wildtype recombinant erythropoietin. Altered ability is defined as ability different from that of the wildtype recombinant erythropoietin ability to regulate the growth and differentiation of red blood cell progenitors. As used herein, altered ability to regulate the growth and differentiation of red blood cell progenitor cells refers to biological activity different from wildtype recombinant erythropoietin

activity (i.e., altered biological activity relative to wildtype recombinant erythropoietin activity). The mutated erythropoietin proteins of the present invention can be secreted in homologous and heterologous expression systems. For example, the mutated erythropoietin proteins of the present invention can be secreted in mammalian, bacterial or yeast expression systems.

The present invention also relates to the modified secretable mutant erythropoietin proteins encoded by the isolated DNA described above. These modified secretable erythropoietin proteins have altered biological activities. For example, the modified secretable mutant erythropoietin may have decreased ability relative to wildtype erythropoietin protein to regulate growth and differentiation of red blood cell progenitor cells. As used herein, decreased ability to regulate growth and differentiation of red blood cell progenitor cells is also referred to as decreased biological activity relative to wildtype erythropoietin activity. Wildtype erythropoietin activity is also referred to herein as biological activity of wildtype erythropoietin. Alternately, a modified secretable mutant erythropoietin protein described herein may exhibit increased heat stability relative to wildtype erythropoietin protein.

The modified erythropoietin proteins described herein comprise an amino acid sequence with at least one amino acid residue different from the amino acid residue present at the corresponding position in Domain 1 in the wildtype erythropoietin. These erythropoietin proteins

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are referred to as modified secretable human recombinant erythropoietin proteins having altered ability (i.e., decreasing or enhancing ability) relative to wildtype erythropoietin protein to regulate the growth and
5 differentiation of red blood cell progenitors.

The term modified, as used herein, includes substitution of a different amino acid residue, or residues, as well as deletion or addition of an amino acid residue, or residues.

10 Until the present invention, mutations within the erythropoietin sequence which result in the alteration of biological activity have also frequently resulted in a concurrent loss of secretability of the protein from transfected cells. This loss of secretability is
15 consistent with a loss of structural integrity.
(Boissel, J-P. and Bunn, H. F., "The Biology of Hematopoiesis", pp. 227-232, John Wiley and Sons, New York (1989)). Now, the sites critical to the maintenance of a stable, biologically active
20 conformation have been identified by means of oligonucleotide-directed mutagenesis and have been found to occur in Domain 1 (amino acids 99-110) (SEQ ID NO: 1) of human recombinant erythropoietin. Modifications of the wildtype erythropoietin have been made and the
25 encoded erythropoietin proteins have been expressed. The resulting mutant erythropoietin proteins described herein have altered erythropoietin regulating activity, as demonstrated in the art-recognized bioassay of Krystal, G., *Exp. Hematol.* 11:649-660 (1983). Activity
30 of the resulting erythropoietin proteins has also been

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evaluated by commercially available radioimmunoassay protocols.

In particular, the arginine 103 site is essential for erythropoietin activity. As shown herein, replacement of the arginine 103 by another amino acid results in a modified erythropoietin with significantly decreased biological activity relative to wildtype erythropoietin activity. Modifications at this site, as well as other sites within Domain 1, can similarly be made to enhance regulating activity, as well as to decrease, or reduce regulating ability.

In another embodiment, the present invention relates to mutant proteins described herein that comprise modified erythropoietin proteins produced by alterations in the 5' and/or 3' noncoding regions of the wildtype gene in addition to mutations in coding regions. Hereinafter, the term modified erythropoietin variant protein will be used to describe these molecules.

These recombinant variant proteins can have altered biological activity. Altered biological activity is defined herein as activity different from that of the wildtype or recombinant protein (e.g., the activity of modified erythropoietin variant proteins to regulate the growth and differentiation of red blood cell progenitors). Modified erythropoietin variant proteins can have increased activity relative to wildtype erythropoietin to regulate growth and differentiation of red blood cell progenitor cells. Alternatively, the erythropoietin variant proteins can have decreased

biological activity relative to the wildtype erythropoietin.

Mutations in noncoding regions of the gene (e.g., 5' untranslated regions or UTR) can lead to differences in RNA translation as described, e.g., in Schultz, D.E., et al., *J. Virol.* 70:1041-1049, 1996; Kozak, M., *J. Mol. Biol.* 235:95-110, 1994; and Kozak, M., *J. Biol. Chem.* 266:19867-19870, 1991. For example, as described in detail in Example 4, computer modeling can be used to predict differences in RNA secondary structure (e.g., free energy of loops and base pairs) following nucleotide alterations in 3' and 5' UTR of the erythropoietin gene. Although secondary structure changes in EPO RNA, following mutations in the 5' or 3' UTR, are used as the specific example, it is understood that the instant invention described herein can be used to produce any suitable polypeptide variant protein. As used herein, the term mutation refers to any alteration in the nucleic acid sequence encoding a polypeptide (e.g., a point mutation; the addition, deletion and/or substitution of one or more nucleotides).

Secondary structure has been shown to be a critical component in determining the rates of translation efficiency of several proteins (Bettany, A.J., et al., *J. Biol. Chem.* 267:16531-16537, 1992; Kozak, M., *J. Mol. Biol.* 235:95-110, 1994). By implication, altered rates of translation may affect posttranslational modifications, for example, glycosylation patterns, and, thus, proper folding of the resulting protein leading to changes in the chemistry, structure and function of the

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protein. The modified erythropoietin variant proteins described herein are unique in that they are composed of mutant proteins produced by alterations in 5' and 3' untranslated (noncoding) regions of the gene.

5 The modified secretable erythropoietin proteins described herein provide useful reagents to further elucidate the structure/function relationship of erythropoietin and its cellular receptor.

Such modified secretable erythropoietin proteins
10 with altered regulating ability can also be used for therapeutic purposes. For example, modified erythropoietin proteins with enhanced biological activity would be a more potent therapeutic, therefore requiring a lower effective dose or less frequent
15 administration to an individual. Erythropoietin proteins with decreased biological activity that still retain their structural integrity and bind to their cognate receptor would be useful to decrease growth and differentiation of red blood cell precursors in certain
20 leukemias and polycythemias. Furthermore, an erythropoietin protein that selectively triggers only certain events within the red blood cell precursor cell would be useful in treating various hematological conditions.

25 Further, it is expected that modified secretable mutant erythropoietin proteins with increased heat stability relative to wildtype erythropoietin proteins would have a longer plasma half-life relative to wildtype erythropoietin proteins. Thus, such modified
30 erythropoietin proteins with increased heat stability

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can be useful therapeutically. For example, modified
secretable mutant erythropoietin proteins with increased
heat stability would be especially important in patients
with a fever and/or experiencing an increased metabolic
5 state.

The present invention also relates to methods of
modifying or altering the regulating activity of a
secretable erythropoietin protein.

This invention further relates to pharmaceutical
10 compositions comprising an effective amount of modified
secretable human recombinant erythropoietin in a
physiologically acceptable carrier.

The present invention also relates to a method of
evaluating a substance for ability to regulate growth
15 and differentiation of red blood cell progenitor cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of the in vitro
mutagenesis protocol. WT = wildtype erythropoietin.

Figure 2 depicts the structure of expression vector
20 pSV-2-erythropoietin.

Figure 3 is a graphic representation of the
specific activities of nine mutant erythropoietin
proteins.

Figure 4 is a graphic representation of the results
25 of monoclonal antibody precipitation of the mutant
erythropoietin proteins.

Figure 5 is a graphic representation of the
activity of heat-denatured wildtype erythropoietin as

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measured by radioimmunoassay (■) and the Krystal bioassay (●).

Figure 6A-6H is a graphic representation of the activity of the 103 mutant erythropoietin proteins as measured by radioimmunoassay (■) and the activity of wildtype erythropoietin (●).

Figure 6A shows the activity of R103A. Figure 6B shows the activity of R103D. Figure 6C shows the activity of R103K. Figure 6D shows the activity of R103E. Figure 6E shows the activity of R103N. Figure 6F shows the activity of R103Q. Figure 6G shows the activity of R103H. Figure 6H shows the activity of R103L.

Figure 7 is a schematic representation describing how differences in mRNA and protein structure; and protein function can result from alterations in the 5' and 3' UTR of a gene.

Figures 8A-C depict the nucleotide sequence of the human erythropoietin gene (SEQ ID NO:23).

Figures 9A-F depict the nucleic acid sequence of nucleotides 401-624 in the 5' untranslated region of the EPO gene (SEQ ID NO:24) (Figure 9A) and five variant sequences (SEQ ID NOS: 25-29) (Figures 9B-9F).

Figures 10A-10E depict the nucleic acid sequence of nucleotides 2773-2972 in the 3' untranslated region of the EPO gene (SEQ ID NO:30) (Figure 10A) and four variant sequences in that region (SEQ ID NOS: 31-34) (Figures 10B-10E).

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DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the identification of amino acid residues of the erythropoietin polypeptide which are critical for its biological activity and secretable properties. These sites have been precisely defined through oligonucleotide-directed mutagenesis and used to create mutant human recombinant erythropoietin proteins which are altered by one, or more, amino acid substitutions and thus differ from wildtype erythropoietin. The term "recombinant", as used herein, means that a host protein is derived from recombinant (e.g., eukaryotic or prokaryotic host cell) expression systems which include, for example, yeast (e.g., *Saccharomyces*), bacteria (such as, *Escherichia* or *Bacillus*), and animal cells including insect or mammalian expression systems. Proteins expressed in most bacterial cultures will be free of glycan. Protein expressed in yeast may have a glycosylation pattern different from protein expressed in mammalian cells.

As used herein, the term nucleotide sequence or nucleic acid sequence refers to a heteropolymer of deoxyribonucleotides (DNA) or ribonucleotides (RNA).

Nucleic acid sequences encoding the proteins provided in this invention can be assembled from DNA, either cDNA or genomic DNA, or RNA, and short oligonucleotide linkers to provide a synthetic nucleic acid sequence which is capable of being expressed in a recombinant transcriptional unit. Homologous nucleic acids, including DNA or RNA, can be detected

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and/or isolated by hybridization (e.g., under high stringency conditions or moderate stringency conditions). "Stringency conditions" for hybridization is a term of art which refers to the conditions of temperature and buffer concentration which permit hybridization of a particular nucleic acid to a second nucleic acid in which the first nucleic acid may be perfectly complementary to the second, or the first and second may share some degree of complementarity which is less than perfect. For example, certain high stringency conditions can be used which distinguish perfectly complementary nucleic acids from those of less complementarity. "High stringency conditions" and "moderate stringency conditions" for nucleic acid hybridizations are explained in several technical protocol reference texts, for example, Ausubel, F.M., et al., *"Current Protocols in Molecular Biology"* (1995), the teachings of which are hereby incorporated by reference. The exact conditions which determine the stringency of hybridization depend not only on ionic strength, temperature and the concentration of destabilizing agents such as formamide, but also on factors such as the length of the nucleic acid sequence, base composition, percent mismatch between hybridizing sequences and the frequency of occurrence of subsets of that sequence within other non-identical sequences. Thus, high or moderate stringency conditions could be determined for detecting the various forms of recombinant erythropoietin.

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By varying hybridization conditions from a level of stringency at which no hybridization occurs to a level at which hybridization is first observed, conditions which will allow a given sequence to hybridize (e.g.,
5 selectively) with the most similar sequences in the sample can be determined.

Exemplary conditions are described in Krause, M.H. and Aaronson, S.A., *Methods in Enzymology*, 200:546-556, 1991.. Also, "*Current Protocols in Molecular Biology*"
10 (*supra*), which describes how to determine washing conditions for moderate or low stringency conditions. Washing is the step in which conditions are usually set so as to determine a minimum level of complementarity of the hybrids. Generally, starting from the lowest
15 temperature at which only homologous hybridization occurs, each °C by which the final wash temperature is reduced (holding SSC concentration constant) allows an increase by 1% in the maximum extent of mismatching among the sequences that hybridize. Generally, doubling
20 the concentration of SSC results in an increase in T_m of -17°C. Using these guidelines, the washing temperature can be determined for high, moderate or low stringency, depending on the level of mismatch sought. For example, in this invention alterations in the noncoding regions
25 of the gene (5' and 3'untranslated regions) may necessitate changes in stringency conditions from low to medium to high depending upon the number of nucleotides that are modified that differ from the condition used to detect wild type versions of the gene. Where

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appropriate the salt concentrations and temperatures will be adjusted accordingly.

IDENTIFICATION OF AMINO ACID RESIDUES OF HUMAN
RECOMBINANT ERYTHROPOIETIN CRITICAL FOR BIOLOGICAL

5 ACTIVITY

Previously, anti-peptide antibodies to several hydrophilic domains of the erythropoietin molecule had demonstrated that antibodies to amino acids 99-110 (Domain 1) and 111-129 (Domain 2) block the hormone's
10 biological activity. Binding of the antibody to a portion of the erythropoietin molecule that participated in receptor recognition would block such recognition, thereby neutralizing erythropoietin's biological activity. (Sytkowski, A. J. and Donahue, K. A., J.
15 *Biol. Chem.* 262:1161-1165 (1987)).

A series of mutants across the 99-129 region was produced by sequentially replacing three amino acids with Glu-Phe. Mutations in amino acid residues 99-110 caused a profound structural change which inhibited
20 secretion of the mutant erythropoietin after biosynthesis. (Chern, Y., et al., *Eur. J. Biochem.* 202:225-229 (1991)). To precisely identify the amino acid site, or sites, critical for receptor recognition and biological activity, amino acids 100-109 were
25 studied by alanine scanning mutagenesis, as described in detail in Example 1.

Briefly, human recombinant erythropoietin cDNA (Powell, J.W., et al., *Proc. Natl. Acad. Sci. USA* 83:6465-6469 (1986)) was inserted into the Phagemid

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vector pSELECT (Promega Corp., Madison, WI) which contains two genes for antibiotic resistance. One of these genes, specific for tetracycline resistance is always functional, while the other, specific for
5 ampicillin resistance, has been inactivated. The single-stranded template for the mutagenesis reaction was prepared by growing cultures of bacteria transformed with the Phagemid and infected with a helper phage. The resulting single-stranded DNA was isolated.

10 Two oligonucleotides were annealed to this recombinant ssDNA template. The first oligonucleotide was an ampicillin repair oligo designed to convert the vector to ampicillin resistance and the second oligonucleotide was a mutagenic oligo designed to change
15 a portion of the erythropoietin cDNA sequence.

Subsequently, the mutant second strand was synthesized in vitro using T4 DNA polymerase and ligated. This DNA was then transformed into a repair minus strain of *E. coli* and these cells were grown in
20 the presence of ampicillin. The phagemid was then harvested and a second round of transformation was carried out and mutants were selected on ampicillin plates. This resulted in the production of a double stranded phagemid containing both the ampicillin
25 resistance gene and the mutated erythropoietin cDNA.

Figure 1 shows the region of the erythropoietin cDNA encoding amino acids 96-113 (SEQ ID NO: 2) and the corresponding wildtype erythropoietin DNA sequence encoding amino acids 96-113 (SEQ ID NO: 3). The column
30 of numbers on the left hand side of Figure 1 indicates

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the amino acid substitution. The only amino acid residue substitutions made were as indicated. The remainder of the human recombinant erythropoietin DNA sequence was not altered. (The remaining, unaltered human recombinant DNA sequence is not shown.) Thus, for example, 100A (SEQ ID NO: 4) indicates that amino acid 100, normally a serine residue, was replaced by alanine, 101A (SEQ ID NO: 5) indicates that glycine 101 was replaced by alanine, and so forth (SEQ ID NOS: 6-16).

Some sites were mutated more than once. For example, amino acid 103 was mutated twice. The first mutation was the substitution of alanine for arginine 103 (SEQ ID NO: 7) and the second substitution was aspartic acid for arginine (SEQ ID NO: 8).

Two double mutants were also produced, 108A/113R (SEQ ID NO: 12) and 109A/113R (SEQ ID NO: 13). In these two instances, amino acids 108 and 109 were each substituted with alanine in the second mutation and the replacement of glycine 113 with arginine was introduced. The changes in nucleotide sequence in each mutagenic oligo are indicated in Figure 1 and Table I (SEQ ID NOS: 4-22). In Table I, the underlined nucleotides are those which differ from the wildtype erythropoietin sequence. A silent mutation designed to introduce a restriction site, Hinf I, allowing convenient initial screening for mutated erythropoietin cDNAs, was also introduced.

In addition, two mutants in the region of the erythropoietin cDNA encoding amino acids 1-26 (the amino-terminus region) were produced. In these two instances, amino acid 14, normally an arginine, was

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replaced either by alanine (14A) or aspartic acid (14D).

Each mutated erythropoietin cDNA was identified by restriction analysis, using standard laboratory protocols, and its structure was confirmed by DNA

5 sequencing. The mutated erythropoietin cDNA was then inserted into the expression vector pSV-2 (Figure 2) using standard laboratory techniques. (Mulligan, R. C., et al. *Nature* 277:108-114 (1979); Sambrook, et al., *"Molecular Cloning: A Laboratory Manual"*, (1989)).

10 As described in detail in Example 2, COS-7 cells were transfected with the pSV-2-erythropoietin constructs. After three days, the supernatant medium was harvested and the biological activity of the mutant erythropoietin proteins and wildtype erythropoietin was
15 measured by the Krystal bioassay (Krystal, G., *Exp. Hematol.* 11:649-660 (1983)). Briefly, the bioassay of Krystal measures the effect of erythropoietin on intact mouse spleen cells. Mice were treated with phenylhydrazine to stimulate production of
20 erythropoietin-responsive red blood cell progenitor cells. After treatment, the spleens were removed, intact spleen cells were carefully isolated and incubated with various amounts of wildtype erythropoietin or the mutant erythropoietin proteins
25 described herein. After an overnight incubation, ^3H thymidine was added and its incorporation into cellular DNA was measured. The amount of ^3H thymidine incorporation is indicative of erythropoietin-stimulated production of red blood cells via interaction of
30 erythropoietin with its cellular receptor. The

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concentration of mutant erythropoietin protein, as well as the concentration of wildtype erythropoietin, was quantified by competitive radioimmunoassay (Incstar, Stillwater, MN). Specific activities were calculated as international units measured in the Krystal bioassay divided by micrograms as measured as immunoprecipitable protein by RIA. Both assays used wildtype recombinant human erythropoietin standardized against the World Health Organization Second International Reference Standard preparation.

Two sets of experiments were performed in order to determine the specific biological activities of these mutant erythropoietin proteins. Specific activities of nine of the mutant erythropoietin proteins (SEQ ID NOS: 4-13) assayed in the first set of experiments are shown in Figure 3. As shown in Figure 3, the specific activities are presented as a percent of the wildtype erythropoietin activity for each mutant erythropoietin. The amino acid replaced by alanine is indicated along the horizontal axis. Table I also shows the specific activities of the nine mutant erythropoietin proteins (SEQ ID NOS: 4-13) as well as nine additional mutant erythropoietin proteins (SEQ ID NOS: 14-22) again assayed in the first set of experiments. The specific activity noted in Table I is also that activity relative to wildtype erythropoietin's activity, which is set at 100%.

As shown in Table I, substitution of alanine for serine 104 decreased activity to approximately 16% of wildtype erythropoietin (SEQ ID NO: 14). Substitution

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of alanine for leucine 105 (SEQ ID NO: 9) reduced the activity to approximately 44 percent of wildtype erythropoietin. Substitution of alanine for leucine 108 (SEQ ID NO: 15) reduced the activity to approximately
5 37% of wildtype erythropoietin.

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TABLE I
ALANINE SCANNING MUTAGENESIS OF AMINO ACIDS
100-109 OF ERYTHROPOIETIN

| SEQ MUTANT | OLIGONUCLEOTIDE | SPECIFIC | |
|---------------|---|----------|--------|
| | | ACTIVITY | ID NO. |
| S100A | GGATAAAGCCGT <u>CG</u> CTGGCCTTCGCAGCCTCAC <u>G</u> ACTCTGCTTCGGG | 107.9% | 4 |
| G101A | GCCGTCAGTG <u>CC</u> CTTCGCAGCCTCAC <u>G</u> ACTCTGCTTCGGG | 126.8% | 5 |
| L102A | GCCGTCAGTGGC <u>G</u> CTCGCAGCCTCACC | 93.3% | 6 |
| R103A | CGTCAGTGGCCTT <u>G</u> CCAGCCTCAC <u>G</u> ACTCTGCTTCGG | 0.0% | 7 |
| R103D | CGTCAGTGGCCTT <u>G</u> A CAGCCTCAC <u>G</u> ACTCTGCTTCGG | 0.0% | 8 |
| L105A | GGCCTTCGCAGC <u>G</u> CCAC <u>G</u> ACTCTGCTTCGGG | 44.0% | 9 |
| T106A | GCCTTCGCAGCCTC <u>G</u> C <u>G</u> ACTCTGCTTCGGGG | 76.9% | 10 |
| T107A | CGCAGCCTCACCGCTCTGCTTCG <u>A</u> GCTCTGCGAGCC | 86.6% | 11 |
| L108A/G113R | GCCTCACCACT <u>G</u> CCCTTCG <u>A</u> GCTCTG <u>G</u> GAGCC | 77.3% | 12 |
| L109A/G113R | CCTCACCACTCTG <u>G</u> CTCGGGCTCTGCG | 84.7% | 13 |
| S104A | GTGGCCTTCGCG <u>CC</u> CTCAC <u>G</u> ACTCTGCTTC | 16.3% | 14 |
| L108A | CCTCACCACT <u>G</u> CGCTTCGAGCTCTGGGAGC | 36.9% | 15 |
| L109A | CCTCACCACTCTG <u>G</u> CTCGGGCTCTGGG | 70.2% | 16 |
| R103N | CGTCAGTGGCCTT <u>A</u> ACAGCCTCAC <u>G</u> ACTCTGCTTCGG | 0.0% | 17 |
| R103E | CGTCAGTGGCCTT <u>G</u> AGAGCCTCAC <u>G</u> ACTCTGCTTCGG | 0.0% | 18 |
| R103Q | CGTCAGTGGCCTT <u>C</u> AGAGCCTCAC <u>G</u> ACTCTGCTTCGG | 0.0% | 19 |
| R103H | CGTCAGTGGCCTT <u>C</u> ACAGCCTCAC <u>G</u> ACTCTGCTTCGG | 0.0% | 20 |
| R103L | CGTCAGTGGCCTT <u>C</u> TACAGCCTCAC <u>G</u> ACTCTGCTTCGG | 0.0% | 21 |
| R103K | CGTCAGTGGCCTG <u>A</u> AGAGCCTCAC <u>G</u> ACTCTGCTTCGG | 10.2% | 22 |

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To further characterize the muteins obtained by substitution of the 103 arginine amino acid residue (SEQ ID NOS: 7, 8 and 17-22), a second set of experiments with COS-7 cells transfected as described in Example 2 with the pSV-2-erythropoietin mutant constructs encoding these muteins was performed. The supernatant medium was again harvested after three days and the biological activity of the mutant erythropoietin proteins was measured by the Krystal bioassay, the concentration of mutant erythropoietin protein was quantified by competitive radioimmunoassay (Incstar, Stillwater, MN) and specific activities (shown in Table II) were calculated as international units measured in the Krystal bioassay divided by micrograms as measured as immunoprecipitable protein by RIA.

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TABLE II

MUTAGENESIS OF AMINO ACID
Arg 103 OF ERYTHROPOIETIN

| SEQ MUTANT | OLIGONUCLEOTIDE | SPECIFIC | |
|---------------|--|----------|--------|
| | | ACTIVITY | ID NO: |
| R103A | CGTCAGTGGCCTTGCCAGCCTCACGACTCTGCTTCGG | 0.0% | 7 |
| R103D | CGTCAGTGGCCTTGACAGCCTCACGACTCTGCTTCGG | 0.0% | 8 |
| R103N | CGTCAGTGGCCTTAACAGCCTCACGACTCTGCTTCGG | 0.0% | 17 |
| R103E | CGTCAGTGGCCTTGAGAGCCTCACGACTCTGCTTCGG | 0.0% | 18 |
| R103Q | CGTCAGTGGCCTTCAGAGCCTCACGACTCTGCTTCGG | 0.0% | 19 |
| R103H | CGTCAGTGGCCTTCAAGAGCCTCACGACTCTGCTTCGG | 1.7% | 20 |
| R103L | CGTCAGTGGCCTTCTCAGCCTCACGACTCTGCTTCGG | 0.4% | 21 |
| R103K | CGTCAGTGGCCTGAAGAGCCTCACGACTCTGCTTCGG | 25.0% | 22 |

As shown in Table II, mutants having arginine 103 substituted by histidine (SEQ ID NO: 20) exhibited decreased activity to approximately 1.7 % of wildtype erythropoietin. Specific activity is again defined as 5 percent activity of wildtype erythropoietin activity. Mutants having arginine 103 substituted by leucine (SEQ ID NO: 21) exhibited decreased activity to approximately 0.4 % of wildtype erythropoietin. Mutants having arginine 103 substituted by lysine (SEQ ID NO: 22) exhibited decreased activity to 10 approximately 25 % of wildtype erythropoietin compared

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to approximately 10 % of wildtype erythropoietin shown previously (compare Table I and Table II).

The results show that these three mutant erythropoietin proteins (SEQ ID NOS: 20-22) have some
5 intrinsic agonist activity (biological activity), thus indicating that the erythropoietin muteins (SEQ ID NOS: 20-22) must bind to the erythropoietin receptor. This phenomenon of weak agonist activity is commonly seen in pharmacologic blockers when tested at high enough
10 concentrations. Thus, it is reasonable to predict that equivalent quantities of these extremely low activity muteins would compete effectively with native erythropoietin and block activity.

As shown in Table II, mutants having arginine 103
15 substituted by alanine (SEQ ID NO: 7), aspartic acid (SEQ ID NO: 8), asparagine (SEQ ID NO: 17), glutamic acid (SEQ ID NO: 18), and glutamine (SEQ ID NO: 19) exhibited essentially no erythropoietin biological activity as was shown previously (Table I). The
20 results of these experiments indicate that amino acid position 103 is important for erythropoietin biological activity. Although all of these mutants were expressed and secreted into culture medium at rates equivalent to that seen for wildtype and other mutants, only very low
25 levels of biological activity were detected or, in some cases, no biological activity was detected. Methods described herein, such as the ex vivo bioassay of Krystal (Krystal, G., *Exp. Hematol.* 11:649-660 (1983)), which is an art-recognized bioassay used to evaluate
30 erythropoietin activity, showed that these inactive

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arginine 103 mutants are reduced in activity by at least a 1000-fold below that of the wildtype human recombinant erythropoietin.

Previously published studies indicated that 5 mutations in the Domain 1 region resulted in biologically inactive muteins. (Chern, Y., et al., *Eur. J. Biochem.* 202:225-229 (1991)). Thus, modified secretable erythropoietin proteins with mutations in the Domain 1 region would not be expected to have 10 enhanced biological activity relative to wildtype erythropoietin proteins. That is, making mutations in this critical and highly conserved region of the erythropoietin protein would not be expected to result in the production of muteins with increased specific 15 activity relative to wildtype erythropoietin proteins. Surprisingly, as shown in Table I, substitution of alanine for serine 100 (SEQ ID NO: 4) and glycine 101 (SEQ ID NO: 5) increased the specific activity of these mutant proteins.

20 To determine if the increased specific activity of the muteins obtained by substitution of alanine for serine 100 (S100A; SEQ ID NO: 4) and glycine 101 (G101A; SEQ ID NO: 5) was statistically significant, a statistical analysis based on the Student-t 25 distribution for small samples was performed. The mean values obtained were compared to that of wildtype erythropoietin activity using the "difference between two sample means" statistic (one-sided). The increased specific activity of G101A over wildtype was found to 30 be statistically significant at the 0.05 level of

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significance. The increased specific activity of S100A was not found to be statistically significant below the 0.010 level of significance.

Additionally, mutants having arginine 14 substituted by alanine (R14A) exhibited decreased activity to approximately 16.4 % of wildtype erythropoietin. Mutants having arginine 14 substituted by aspartic acid (R14D) exhibited decreased activity to approximately 3.9 % of wildtype erythropoietin.

10 STRUCTURAL INTEGRITY OF MUTANT ERYTHROPOIETIN PROTEINS

Previously published studies indicated that mutations in the Domain 1 region in which a group of three amino acids was deleted and replaced with Glu-Phe, caused pronounced structural changes in the molecule. (Chern, Y., et al., *Eur. J. Biochem.* 202:225-229 (1991)). These structural changes were accompanied by lack of secretion of the mutant erythropoietin from the transfected COS-7 cells. Surprisingly, this phenomenon was not observed with the more subtle mutations of the present invention. Thus, the mutant erythropoietin proteins described herein provide structurally intact (i.e., with the proper biological conformation) mutant erythropoietin proteins.

25 Assessment of the structural integrity of the mutated erythropoietin proteins of the instant invention was performed by a series of immunoprecipitation experiments using anti-peptide

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monoclonal antibodies to two domains of the protein, as described in Example 3.

Briefly, the first monoclonal antibody recognizes an epitope within amino acids 1-26 of erythropoietin.

5 The other monoclonal antibodies recognize distinct epitopes within amino acids 99-129. It is known that a gross change in the tertiary structure of erythropoietin would result in an inability of one or more of the monoclonal antibodies to recognize the
10 erythropoietin molecule. For example, it has been demonstrated that radio-iodination of erythropoietin in the presence of chloramine-T denatures the molecule, resulting in loss of biological activity and corresponding loss of recognition by monoclonal
15 antibody.

Figure 4 shows mutant erythropoietin protein precipitated as percent of control of wildtype erythropoietin precipitated using three monoclonal antibodies designated across the horizontal axis, 1-26,
20 99-129 α and 99-129 β . The three erythropoietin proteins examined were the wildtype erythropoietin, the 103 alanine mutant and the 103 aspartic acid mutant. As seen on the left side of the graph, monoclonal 1-26 recognized each of the three recombinant erythropoietin
25 proteins with equal efficiency, indicating that mutation of amino acid 103 to either alanine or aspartic acid did not result in a gross distortion of erythropoietin's conformation.

Similarly, as shown in the center of the graph,
30 monoclonal 99-129 α also recognized the wildtype 103

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alanine mutant and 103 aspartic acid mutant with no statistically significant difference among them. This indicates that the conformation within the amino acids 99-129 is similar among the three recombinant erythropoietin proteins.

Lastly, as shown on the right side of the graph, monoclonal 99-129 β recognized both mutant erythropoietin proteins with approximately half the efficiency as it recognized the wildtype erythropoietin. This is consistent with the subtle structural change introduced by a single amino acid mutation. Taken together, it is reasonable to assume that the inactive point mutants, 103 alanine and 103 aspartic acid, are not grossly denatured.

15 HEAT STABILITY OF MUTANT ERYTHROPOIETIN PROTEINS

A previously published study indicated that recombinant human erythropoietin aggregates as temperature rises. (Endo, Y., et al., *J. Biochem.* 112(5):700-706 (1992)). Most of the erythropoietin molecules within these multimeric aggregates (twenty erythropoietin molecules per aggregate) would almost certainly not be detectable by antibodies in a radioimmunoassay (RIA). Surprisingly, heat reduced the RIA detection of wildtype erythropoietin much more rapidly than the more stable mutants of the present invention. Thus, some of the mutant erythropoietin proteins described herein demonstrate increased heat stability relative to the wildtype erythropoietin protein.

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Assessment of the heat stability of the mutated erythropoietin proteins of the instant invention was performed by comparing *in vitro* biological activity with antibody reactivity. Briefly, aliquots of 5 conditioned medium from erythropoietin cDNA-transfected COS cells were incubated at 56°C for specified time intervals. The samples were cooled on ice and a fraction of each was assessed for biological activity in the Krystal bioassay. The remainder was split into 10 two fractions and erythropoietin protein was quantified by radioimmunoassay using the commercially available INCSTAR RIA kit. The results are given in terms of the percent biological activity remaining or percent protein immunoprecipitated after heat treatment 15 compared to untreated samples.

Wildtype erythropoietin exhibits a time-dependent decrease in biological activity when incubated at 56°C or above (Figure 5); Tsuda, E., et al., *Eur. J. Biochem.* 188:405-411 (1990). Interestingly, a 20 corresponding decrease in the ability of the commercial radioimmunoassay's antibodies to recognize this heat-denatured erythropoietin was also observed (Figure 5). This observation was quite reproducible and enabled the use of the RIA to measure the heat stability of the 25 inactive R103A erythropoietin compared to that of wildtype erythropoietin. As seen in Figure 6A, the heat denaturation curves of R103A and wildtype erythropoietin are essentially identical.

To confirm that this heat stability comparison is 30 sensitive to mutations in this region of

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erythropoietin, the effect of the aspartic acid substitution (R103D) on the protein's stability was evaluated. The introduction of a negatively charged amino acid residue would reasonably be more
5 structurally disruptive to the molecule than an alanine, and thus be more likely to alter the protein's heat-denaturation curve. The heat stability of R103D was markedly different (i.e., greater) than that of wildtype erythropoietin and R103A, as anticipated
10 (Figure 6B).

To further characterize the nature of the interaction between amino acid residue 103 and the erythropoietin receptor, site-directed mutagenesis was used to produce erythropoietin analogs with altered
15 side chain properties at this position. Arginine was substituted with histidine (R103H), lysine (R103K), asparagine (R103N), glutamine (R103Q), leucine (R103L) and glutamic acid (R103E) to generate 6 new altered erythropoietin molecules. Culture supernatants of
20 cells transfected with these constructs in a first set of experiments were tested in the Krystal bioassay and the heat stability assay for biological activity and structural stability, respectively.

The heat denaturation curve of R103K was
25 essentially identical to that generated for the wildtype protein. Interestingly, the heat denaturation curve for R103E was notably different from that of wildtype, and very similar to that of R103D. The other
4 mutants had denaturation kinetics intermediate to
30 that of these two proteins. (See Figures 6C-6H).

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PRODUCTION OF ADDITIONAL ERYTHROPOIETIN PROTEINS HAVING ALTERED BIOLOGICAL ACTIVITY

As a result of the identification of sites which are critical to erythropoietin activity in terms of the 5 amino acid residue present and which can be altered to produce a mutated sequence which has altered biological activity but retains its structural integrity, it is now possible to produce modified secretable human recombinant erythropoietin proteins whose ability to 10 regulate the growth and differentiation of red blood cell progenitors is altered (i.e., whose ability to regulate red blood cell progenitors is different from that of the corresponding wildtype human recombinant erythropoietin). These modified human recombinant 15 erythropoietin proteins can be secreted in homologous or heterologous expression systems.

As described in the previous sections and in the Examples, such sites have been identified by oligonucleotide-directed mutagenesis and used to create 20 mutant erythropoietin which resulted in substitution of amino acids at positions 100-109 within Domain 1 (SEQ ID NO: 1), as represented in Figure 1 (SEQ ID NOS: 4-13) and Table I (SEQ ID NOS: 4-16). The data indicate that arginine 103 is critical for erythropoietin's 25 biological activity. Additionally, serine 104, leucine 105 and leucine 108 appear to play a role, as indicated by the decreased biological activity of these mutants as measured in the above-described bioassays.

It is important to note that the ability of 30 erythropoietin to regulate growth and differentiation

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of red blood cell progenitors depends on the ability of erythropoietin to bind to its cellular receptor. Importantly, the mutations described herein do not disrupt the structural integrity of the erythropoietin protein, as evidenced by the fact that the mutated protein is secreted. That is, as the data presented herein indicates, these mutant erythropoietin proteins retain their biological conformation. These results also indicate that Domain 1 amino acids 99-110 very likely participate in receptor recognition and activation.

Moreover, as the data presented herein indicates, some mutant erythropoietin proteins also demonstrate increased heat stability relative to the wildtype erythropoietin, even though the biological activity of the mutant has been significantly decreased.

Substitution of alanine at arginine 103 produced erythropoietin mutants with no detectable erythropoietin activity as measured by standard techniques. Mutations at serine 104, leucine 105 and leucine 108 also significantly decreased biological activity relative to wildtype erythropoietin activity. In a similar manner, other changes at one or more of these critical sites can result in reduction of erythropoietin activity. Conversely, amino acid residues can be introduced at these critical sites to produce modified secretable human recombinant erythropoietin proteins with enhanced biological activity relative to wildtype erythropoietin activity.

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Conservative substitutions can be made at one or more of the amino acid sites within residues 100-109 of the molecule. For example, alanine and aspartic acid have been used to replace arginine 103. Substitution
5 of these amino acids by other amino acids of the same type (i.e., a positively charged, or basic, amino acid for a positively charged, or basic, one, or an acidic amino acid for an acidic one) as that present at that specific position can be made and the effect on
10 erythropoietin's ability to regulate the growth and differentiation of red blood cell progenitors can be determined, using the methods described herein.

Substitutions at these critical sites, alone or in combination, of amino acids having characteristics
15 different from those of amino acids whose presence at those sites has been shown to eliminate or reduce erythropoietin activity can also be made and their effect on activity assessed as described above. In particular, substitutions of some, or all, of the amino
20 acids at one, or more, of these critical sites which result in modified secretable erythropoietin proteins with enhanced erythropoietin activity can be made. Using the techniques described herein, erythropoietin proteins having enhanced biological activity can be
25 identified.

In addition, more radical substitutions can be made. For example, an amino acid unlike the residue present in the corresponding position in the wildtype sequence is substituted for the residue in wildtype
30 erythropoietin (e.g., a basic amino acid is substituted

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for an acidic amino acid). Each resulting mutant is then evaluated using the anti-erythropoietin immunoprecipitation techniques and biological activity assays as described.

5 As a result, modified secretable human recombinant erythropoietin proteins having enhanced erythropoietin activity or increased heat stability can be identified. Similar techniques can be used to identify additional critical sites and subsequently, to make substitutions
10 and evaluate their effects on erythropoietin regulating activity.

The present invention also relates to modified erythropoietin variant mutant proteins encoded by nucleic acids that contain alterations in noncoding
15 regions of the gene in addition to mutations in coding regions as described above.

The variant nucleic acid molecules encoding, for example, modified erythropoietin variant mutant proteins created by altering the 3' and/or 5' UTR of
20 the erythropoietin gene, would preferably contain regulatory sequences. Regulatory sequences include all cis-acting elements that control transcription and regulation such as, promoter sequences, enhancers, ribosomal binding sites, and transcription binding
25 sites. Selection of the promoter will generally depend upon the desired route for expressing the protein. For example, where the mutant erythropoietin variant protein is to be expressed in a recombinant eukaryotic or prokaryotic cell, the selected promoter is recognized
30 by the host cell. A suitable promoter which can be

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used can include the native promoter for the binding moiety which appears first in the construct.

The elements which comprise the nucleic acid molecule can be isolated from nature, modified from native sequences or manufactured *de novo*, as described, for example, in the several art-recognized laboratory technical protocol texts such as Sambrook, et al., "Molecular Cloning: A Laboratory Manual," (1989) and Ausubel, et al. "Current Protocols in Molecular Biology," (1995). The elements can then be isolated and fused together by methods known in the art, such as exploiting and manufacturing compatible cloning or restriction sites.

The nucleic acid molecules encoding modified erythropoietin variant proteins can be inserted into a construct which can, optionally, replicate and/or integrate into a recombinant host cell, by known methods which may vary depending upon the form of the recombinant erythropoietin mutein which is expressed. The host cell can be a eukaryotic or prokaryotic cell and includes, for example, pichia expression systems, yeast (such as, *Saccharomyces*), bacteria (such as, *Escherichia* or *Bacillus*), animal cells or tissue, including insect (such as, *Spodoptera frugiperda* 9) or mammalian cells (such as, somatic or embryonic human cells, Chinese hamster ovary cells, HeLa cells, human 293 cells, monkey kidney COS-7 cells, baby hamster kidney BHK cells, C127 cells, etc.). The selection of the host cell governs the posttranslational modifications that may occur. For instance,

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glycoproteins could be expressed in mammalian, insect, or yeast cells whereas nonglycosylated protein could be expressed in bacteria.

In addition, the selection of the appropriate host cell may differ when expressing recombinant modified erythropoietin variant proteins manufactured by alterations in the noncoding regions of the gene. (Schultz, et al., *J. Virol.* 70:1041-1049, 1996).

The nucleic acid molecule can be incorporated or inserted into the host cell by known methods. Examples of suitable methods of transfecting or transforming cells include calcium phosphate precipitation, electroporation, microinjection, infection, lipofection and direct uptake. Methods for preparing such recombinant host cells are described in more detail in several technical books, for example, Sambrook, et al., (*supra*) and Ausubel, et al. (*supra*).

The host cells are maintained under suitable conditions for expressing and recovering the recombinant modified erythropoietin variant protein. Generally, the cells are maintained in a suitable buffer and/or growth medium or nutrient source for growth of the cells and expression of the gene product(s). The growth media are generally known in the art and include sources of carbon, nitrogen and sulfur. Examples include Dulbeccos modified Eagles media (DMEM), RPMI-1640, M199 and Grace's insect media. The selection of a buffer is not critical to the invention. The pH which can be selected is generally

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one tolerated by or optimal for growth for the host cell.

The cell is maintained under a suitable temperature and atmosphere. For example, an aerobic host cell is maintained under aerobic atmospheric conditions or other suitable conditions for growth. The temperature should also be selected so that the host cell tolerates the process and can be, for example, between about 27°C and 40°C.

10 APPLICATIONS OF MODIFIED SECRETABLE ERYTHROPOIETIN PROTEINS HAVING ALTERED BIOLOGICAL ACTIVITY

As described above, arginine 103 is essential for erythropoietin's biological activity. Additionally, serine 104, leucine 105 and leucine 108 also appear to play a significant role in biological activity. Furthermore, these subtle point mutations do not compromise the structural integrity, (i.e., secretability) of the erythropoietin molecule. Since these described muteins have some intrinsic biological activity as detected by the assays described herein, albeit significantly reduced from wildtype erythropoietin, it is reasonable to assume that they do bind to the erythropoietin receptor. Thus, it is reasonable to assume that the mutant erythropoietin proteins will be recognized by the erythropoietin cellular receptor in essentially the same manner as the wildtype erythropoietin.

Modified secretable human recombinant erythropoietin proteins of the present invention can be

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used for therapy and diagnosis of various hematologic conditions. For example, an effective amount of modified secretable recombinant erythropoietin with enhanced biological activity to regulate the growth and differentiation of red blood cell progenitors can be used therapeutically (*in vivo*) to treat individuals who are anemic (e.g. as a result of renal disease, chemotherapy, radiation therapy, or AIDS). An effective amount of modified secretable human recombinant erythropoietin protein, as defined herein, is that amount of modified secretable erythropoietin protein sufficient to regulate growth and differentiation of red blood cell progenitor cells. For example, modified secretable erythropoietin protein with increased regulatory ability will bind to the erythropoietin receptor and stimulate the growth and differentiation of red blood cell progenitor cells. The modified secretable erythropoietin with enhanced biological activity would be more potent than the wildtype erythropoietin. Thus, to increase red blood cell growth and differentiation in anemic conditions, a lower effective dose or less frequent administration to the individual would be required.

Modified secretable erythropoietin with altered regulating activity can also be used to selectively trigger only certain events regarding the growth and differentiation of red blood cell precursors. For example, it has recently been shown that binding of erythropoietin to its receptor generates two distinct chemical signals in cells, a protein kinase C dependent

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activation of the proto-oncogene c-myc and a phosphatase mediated signal to c-myb. (Spangler, R., et al., *J. Biol. Chem.* 266:681-684 (1991); Patel, H. R. and Sytkowski, A. J., Abstract 1208, *Blood* 78(10) 5 Suppl. 1 (1991)). Thus, a modified secretable erythropoietin can be used to selectively activate either the protein kinase C or the phosphatase pathways.

An effective amount of modified secretable 10 erythropoietin with decreased biological activity relative to wildtype erythropoietin activity, (i.e., reduced biological activity or no detectable biological activity), can be used to treat individuals with various erythroleukemias. In this case, an effective 15 amount of modified secretable erythropoietin protein with decreased regulatory ability will bind to the erythropoietin cellular receptor. However, upon the mutant erythropoietin protein binding to the receptor, it is reasonable to predict that the mutant protein 20 lacks ability to trigger subsequent erythropoietin events. It is further reasonable to predict that, because the mutant erythropoietin does bind to the receptor, it prevents wildtype erythropoietin from binding to the receptor (i.e., competitively inhibits 25 the binding of wildtype erythropoietin). Thus, the red blood cell progenitors do not proliferate and/or differentiate.

The mutant erythropoietin proteins of the present invention are secretable, indicating that they retain 30 their structural integrity, and thus fully participate

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in receptor recognition and binding. The initial interaction of a hormone with its cognate receptor might be expected to result in further conformational changes of the hormone ligand, thereby stabilizing the hormone/receptor complex and allowing the formation of higher ordered complexes. However, if a modified erythropoietin protein of the present invention, with no detectable erythropoietin activity, binds to its receptor, it is reasonable to assume that the subsequent events triggered by receptor binding will be altered or inhibited. Therefore, it is also reasonable to assume that growth and differentiation of red blood cell progenitor cells will be altered or inhibited, thereby inducing a remission in a red blood cell leukemia.

Recently, a constitutively active (hormone independent) form of the murine erythropoietin receptor was isolated. (Watowich, S. S., *Proc. Natl. Acad. Sci. USA* 89:2140-2144 (1992)). It has also been shown that the envelope glycoproteins of certain murine viruses bind to and activate the murine erythropoietin receptor. (Yoshimura, A., *Proc. Natl. Acad. Sci. USA* 87:4139-4143 (1990)). Thus, erythropoietin-independent activation (constitutive activation) of the erythropoietin receptor resulting in red blood cell proliferation in a mammal has been demonstrated. It is reasonable to predict that similar constitutive activation would occur in humans, (for example, a virus similar to Rauscher or Friend virus) may constitutively activate the human erythropoietin receptor also.

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resulting in proliferation of red blood cell progenitors. A modified secretable erythropoietin, which retains its structural integrity to bind to the receptor, yet does not activate red blood cell proliferation, would be useful as an antagonist to block such constitutive activation. Moreover, modified secretable erythropoietin proteins with increased stability would provide long-acting erythropoietin antagonists.

5 proliferation, would be useful as an antagonist to block such constitutive activation. Moreover, modified secretable erythropoietin proteins with increased stability would provide long-acting erythropoietin antagonists.

10 Modified secretable erythropoietin would be useful to treat other various medical disorders. For example, polycythemia vera is characterized by uncontrollable proliferation of red blood cells and is currently treated by chemotherapy, radiation or phlebotomy. The

15 increased number of red blood cells increases blood viscosity, leading to a hypertensive condition that can result in a stroke. It is reasonable to predict that an antagonist of erythropoietin, which binds to the receptor and blocks activation, would be a useful, non-

20 invasive treatment.

Likewise, individuals with cyanotic heart disease often have a hyper-erythropoietin condition, leading to increased erythrocyte proliferation. Also, renal disease patients that are being treated with wildtype

25 erythropoietin may experience an overdose. Once the wildtype erythropoietin has been administered, it continues to act. Thus, in these cases, it would be useful to administer a modified secretable erythropoietin with decreased activity to block the

30 effects of the endogenous and exogenous erythropoietin.

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Furthermore, certain hemolytic anemias, such as sickle cell anemia and thalassemia, result in rapid destruction of red blood cells. The body responds by increasing the levels of erythropoietin produced to
5 stimulate red blood cell production. However, the red blood cells produced carry defective hemoglobin. It would be useful to use a modified secretable erythropoietin to reduce production of defective erythrocytes while another form of therapy is used to
10 stimulate normal hemoglobin synthesis.

Erythropoietin has a relatively short plasma half-life (Spivak, J.L. and Hogans, B.B., *Blood* 73: 90-99 (1989); McMahon, F.G., et al., *Blood* 76: 1718-1722 (1990)), therefore, therapeutic plasma levels are
15 rapidly lost, and repeated intravenous administrations must be made. Although the mechanisms responsible for this relatively short plasma half-life are not well understood, inactivation due to heat denaturation/aggregation is likely to play a role. A
20 previously published study indicated that erythropoietin in human serum is susceptible to inactivation by heat. (Elder, G.E., et al., *Blood Cells* 11: 409-419 (1986)). Thus, it is reasonable to predict that modified secretable erythropoietin with
25 increased heat stability relative to wildtype erythropoietin would have a longer plasma half-life relative to wildtype erythropoietin and thus, be useful therapeutically. This may be especially important in patients with a fever and/or an increased metabolic
30 state.

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It is also reasonable to predict that modified secretable erythropoietins with enhanced biological activity relative to wildtype erythropoietin would require a smaller quantity relative to wildtype erythropoietin to achieve a specified level of biological activity. This enhanced biological activity indicates that an effective amount of modified erythropoietin with enhanced biological activity is substantially less than a comparable effective amount of wildtype erythropoietin. The effective amount of modified erythropoietin with enhanced biological activity is defined herein as the amount of modified erythropoietin required to elicit an erythropoietin response, as indicated by increased growth and/or differentiation of erythrocytic precursor cells. Further, the effective amount of modified erythropoietin with enhanced biological activity will require less frequent administration than an equivalent amount of wildtype erythropoietin. For example, if an effective dose of erythropoietin is typically administered three times a week, modified erythropoietin with enhanced biological activity will only need to be administered once a week. Thus, a reduced quantity of modified secretable erythropoietin with enhanced biological activity would be necessary over the course of treatment than would be necessary if wildtype erythropoietin were used.

Modified secretable erythropoietin may be administered to individuals parenterally or orally. The modified secretable erythropoietin proteins of this

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invention can be employed in admixture with conventional pharmaceutically acceptable carriers. Suitable pharmaceutical carriers include, but are not limited to, water, salt solutions and other 5 physiologically compatible solutions. The modified secretable erythropoietin proteins of the present invention may be administered alone, or combined with other therapeutic agents.

It will be appreciated that the amount of modified 10 secretable erythropoietin administered to an individual in a specific case will vary according to the specific modified secretable erythropoietin protein being utilized, the particular compositions formulated, and the mode of application. Dosages for a given 15 individual can be determined using conventional considerations such as the severity of the condition, body weight, age and overall health of the individual.

Modified secretable erythropoietin can also be used for diagnostic purposes. For example, it can be 20 used in assay procedures for detecting the presence and determining the quantity, if desired, of erythropoietin receptor. A modified secretable erythropoietin with enhanced activity would be useful to increase the sensitivity and decrease the incubation times of such 25 assays. It can also be used in in vitro binding assays to determine the effect of new drugs on the binding of erythropoietin protein to its receptor.

Modified secretable erythropoietin proteins described herein also provide useful research reagents 30 to further elucidate the role of erythropoietin in

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erythropoiesis, as well as the structure/function relationship of erythropoietin and its cellular receptor. For example, modified secretable erythropoietin proteins may be useful for evaluating a substance for ability to regulate growth and differentiation of red blood cell progenitor cells. A reasonable indication of the ability of a substance to regulate growth and differentiation of red blood cell progenitor cells is the extent of binding of the substance to the erythropoietin receptor. The term, extent of binding, as used herein, is defined to mean the amount of substance bound to the receptor (e.g., the percent of substance bound to the receptor as compared to a control substance that binds at approximately 100 percent, or alternately, the specific activity of the test substance). A method for evaluating a substance for ability to regulate growth and differentiation of red blood cell progenitor cells can comprise comparing the extent of binding to the erythropoietin receptor of the substance to be evaluated with the extent of binding to the erythropoietin receptor of a modified secretable mutant erythropoietin protein. If the extent of binding to the erythropoietin receptor of the test substance (i.e., the substance to be evaluated) is comparable to the extent of binding to the erythropoietin receptor of the modified secretable mutant erythropoietin protein, then the extent of binding of the test substance is an indication that the ability of the substance to regulate growth and differentiation of red blood cell

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progenitor cells is of approximately the same ability as the modified secretable mutant erythropoietin. For example, if the specific activity of a test peptide is 25.0%, it is reasonable to assume that the test peptide
5 has the ability to regulate growth and differentiation of red blood cell progenitor cell comparable to the R103K modified erythropoietin.

The term substance, as used herein, is defined to include proteins, e.g., analogues of wildtype
10 erythropoietin, erythropoietin protein fragments, other proteins or peptides, and drugs.

The extent of binding to the erythropoietin receptor can be determined by using any of a number of methods familiar to those of skill in the art. For
15 example, methods such as those described in Yonekura, S. et al., *Proc. Natl. Acad. Sci. USA* 88:1-5 (1991); Chern, Y. et al., *Blood* 76(11):2204-2209 (1990); and Krystal, G., *Exp. Hematol.* 11:649-660 (1983), the teachings of which are incorporated herein by
20 reference, may be used.

The modified erythropoietin mutant proteins of the invention produced, for example, by altering the 5' and/or 3' UTR, can be used as therapeutics for delivery to individuals having diseases or conditions that are
25 associated with deficiencies or abnormalities of the proteins described herein. The retention and/or deletion of nucleotides in the UTR of the erythropoietin gene can produce heterologous therapeutic proteins. Heterologous proteins are herein

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defined as proteins which do not exist in nature and exhibit a range of therapeutic effects.

Recombinant erythropoietin proteins with therapeutic value are known in the art. Examples include Lin (U.S. Patent No. 4,703,008); Sytkowski and Grodberg (U.S. Patent No. 5,614,184); Sytkowski (U.S. Patent No. 5,580,853); and Powell (U.S. Patent No. 5,688,679): the contents of which are incorporated herein by reference.

10 For example, the modified erythropoietin proteins described herein can be employed in any method where EPO would be effective, and in particular in methods where other man-made erythropoietin proteins have not produced any clinically beneficial effect (e.g.,
15 increasing red blood cells in an anemic patient). The mode of erythropoietin administration to patients is preferably at the location of the target cells. As such, the administration can be by injection. Other modes of administration (parenteral, mucosal, systemic,
20 implant, intraperitoneal, etc.) are generally known in the art and, for erythropoietin, can be determined, for example, as described in U.S. Patent No. 5,614,184. The recombinant erythropoietin proteins can, preferably, be administered in a pharmaceutically
25 acceptable carrier, such as saline, sterile water, Ringer's solution, and isotonic sodium chloride solution.

The activity of modified erythropoietin proteins, including variants produced by alterations in the 5'
30 and/or 3' UTR, can be tested, for example, in

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pharmacological differences. Accordingly, the activity of modified erythropoietin proteins can be evaluated therapeutically. For example, pharmacological differences in the secreted and purified erythropoietin manufactured by the disclosed method compared to other man-made or naturally occurring erythropoietins can include:

1. An increase or decrease in the potency when administered to patients in human clinical trials. The difference can be in the required initial dose as well as maintenance doses. A relative potency factor can be evaluated for the modified erythropoietin proteins.
2. A reduction or increase in potential side effects in patients may reflect altered activities of the modified erythropoietin proteins. For example, differences can be manifested as an increase or decrease in blood pressure which can be of extraordinary significance in designing treatment regimens for certain high risk patients like dialysis patients who are, in any case, severely ill.
3. A difference in the time lag between the effect of increasing red blood cells in the patient's serum after administration of the modified erythropoietin proteins. This time-lag has the consequence that the desired therapeutic effect is either accelerated or

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delayed significantly compared to other forms of erythropoietin. A decrease in the time lag would be a desirable therapeutic effect by resulting in a faster benefit to the patient.

- 5 4. The ability of a patient to tolerate one form of erythropoietin and not another. If a patient can not tolerate one form of a modified erythropoietin mutant protein over
10 another, this noncompatibility can indicate therapeutic differences which in turn can reflect structural, biochemical and biological modifications in the various forms of the modified erythropoietin proteins.
- 15 5. An increase in the circulating half-life of EPO in patients which can result in less frequent injections or smaller doses of EPO having to be administered. A prolonged half-life would not only be therapeutically
20 beneficial, but also diminish health care costs in the treatment of chronically ill patients.

Thus, differences in the pharmaceutical characteristics of modified erythropoietin proteins can
25 result in variations in therapeutic effects (e.g., the production of reticulocytes and red blood cells and an increase in hemoglobin synthesis and iron uptake). For example, a difference in the inherent potency which would result in lower bioloads inflicted on the

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patient's body by administering modified erythropoietin protein which leads to an absence or drastic lowering of side effects (which may endanger the patient's life or make it impossible to administer one form of
5 erythropoietin) is particularly important in high risk patients (e.g., patients with kidney disorders) who are at high risk for hypertension, myocardial infarct or stroke.

Thus, retention, deletion, point mutation or
10 substitution in the 5' and/or 3' UTR sequences of a modified erythropoietin mutein gene fragment can ultimately influence the final structure and chemistry of the protein expressed and secreted by a host cell transfected with that gene fragment. As a consequence
15 the resulting expressed modified erythropoietin mutein protein can exhibit varying biological parameters which can be assessed using bioassays and in therapeutics.

This invention will now be illustrated by the following Examples, which are not intended to be
20 limiting in any way.

EXAMPLE 1

OLIGONUCLEOTIDE-DIRECTED MUTAGENESIS OF HUMAN RECOMBINANT ERYTHROPOIETIN

The oligonucleotide-directed mutagenesis used to
25 prepare the modified secretable human recombinant erythropoietin proteins of the present invention was performed using the Altered Sites™ In Vitro Mutagenesis System (Promega Corporation of Madison, WI). The Altered Sites™ system consists of a unique mutagenesis

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vector and a simple, straightforward procedure for selection of oligonucleotide-directed mutants. The system is based on the use of a second mutagenic oligonucleotide to confer antibiotic resistance to the mutant DNA strand. The system employs a phagemid vector, pSELECT™-1, which contains two genes for antibiotic resistance. One of these genes, for tetracycline resistance, is always functional. The other, for ampicillin resistance, has been inactivated.

10 An oligonucleotide is provided which restores ampicillin resistance to the mutant strand during the mutagenesis reaction. This oligonucleotide is annealed to the single-stranded DNA (ssDNA) template at the same time as the mutagenic oligonucleotide and subsequent

15 synthesis and ligation of the mutant strand links the two. The DNA is transformed into a repair minus strain *E. coli*, or other suitable host, and the cells are grown in the presence of ampicillin, yielding large numbers of colonies. A second round of transformation

20 in JM109, or a similar host, ensures proper segregation of mutant and wild type plasmids and results in a high proportion of mutants.

The pSELECT-1 plasmid is a phagemid, defined as a chimeric plasmid containing the origin of a single-

25 stranded DNA bacteriophage. This phagemid produces ssDNA upon infection of the host cells with the helper phage R408 or M13KO7. The vector contains a multiple cloning site flanked by the SP6 and T7 RNA polymerase promoters and inserted into the lacZ α -peptide.

30 Cloning of a DNA insert into the multiple cloning site

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results in inactivation of the α -peptide. When plated on indicator plates, colonies containing recombinant plasmids are white in a background of blue colonies. The SP6 and T7 promoters may be used to generate high specific activity RNA probes from either strand of the insert DNA. These sites also serve as convenient priming sites for sequencing of the insert. The pSELECT-1 vector carries gene sequences for both ampicillin and tetracycline resistance. However, the plasmid is ampicillin sensitive because a frameshift was introduced into this resistance gene by removing the Pst I site. Therefore, propagation of the plasmid and recombinants is performed under tetracycline selection.

15 The pSELECT-Control vector provides a convenient white/blue positive control for mutagenesis reactions. This vector was derived from the pSELECT-1 vector by removing the Pst I site within the polylinker. The resultant frameshift in the lac α -peptide inactivated β -galactosidase and led to a white colony phenotype on indicator plates. A lacZ repair oligonucleotide (supplied with the system) may be used to introduce a four base insertion which corrects the defect in the lacZ gene and restores colony color to blue. The fraction of blue colonies obtained is an indication of the mutagenesis efficiency. When the lacZ repair oligonucleotide is used in combination with the ampicillin repair oligonucleotide to correct this defect, 80-90% of the ampicillin resistant colonies are

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blue. When the lacZ repair oligonucleotide is used alone, a mutagenesis efficiency of only 2-5% is seen.

The mutagenic oligonucleotide must be complementary to the single-stranded target DNA. The 5 ssDNA produced by the pSELECT-1 phagemid is complementary to the lacZ coding strand.

The stability of the complex between the oligonucleotide and the template is determined by the base composition of the oligonucleotide and the 10 conditions under which it is annealed. In general, a 17-20 base oligonucleotide with the mismatch located in the center will be sufficient for single base mutations. This gives 8-10 perfectly matched nucleotides on either side of the mismatch. For 15 mutations involving two or more mismatches, oligonucleotides of 25 bases or longer are needed to allow for 12-15 perfectly matched nucleotides on either side of the mismatch.

Routinely, oligonucleotides can be annealed by 20 heating to 70°C for 5 minutes followed by slow cooling to room temperature.

DNA to be mutated is cloned into the pSELECT-1 vector using the multiple cloning sites. The vector DNA is then transformed into competent cells of JM109, 25 or a similar host, and recombinant colonies are selected by plating on LB plates containing 15µg/ml tetracycline, 0.5mM IPTG, and 40µg/ml X-Gal. After incubation for 24 hours at 37°C, colonies containing recombinant plasmids will appear white in a background 30 of blue colonies.

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To produce single-stranded template for the mutagenesis reaction, individual colonies containing pSELECT-Control or recombinant pSELECT-1 phagemids are grown and the cultures are infected with helper phage 5 as described below. The single-stranded DNA produced is complementary to the lacZ coding strand and complementary to the strand of the multiple cloning site. Two helper phages R408 and M13KO7 can be used to provide the greatest latitude in optimizing ssDNA 10 yields.

PROTOCOL

1. Prepare an overnight culture of cells containing pSELECT™-1 or pSELECT™-Control phagemid DNA by picking individual tetracycline resistant colonies 15 from a fresh plate. Inoculate 1-2ml of TYP broth (Promega) containing 15µg/ml tetracycline and shake at 37°C.
2. The next morning inoculate 5ml of TYP broth containing 15µg/ml tetracycline with 100µl of the 20 overnight culture. Shake vigorously at 37°C for 30 minutes in a 50ml tube.
3. Infect the culture with helper phage R408 or M13KO7 at an m.o.i. (multiplicity of infection) of 10 (i.e., add 10 helper phage particles per cell). 25 For the helper phages supplied with this system, add 40µl. Continue shaking for 6 hours to overnight with vigorous agitation.

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4. Harvest the culture supernatant by pelleting the cells at 12,000 x g for 15 minutes. Pour the supernatant into a fresh tube and spin again for 15 minutes.
5. Precipitate the phage by adding 0.25 volume of phage precipitation solution (Promega) to the supernatant. Leave on ice for 30 minutes, then centrifuge for 15 minutes at 12,000 x g. Thoroughly drain the supernatant.
- 10 6. Resuspend the pellet in 400 μ l of TE buffer (Promega) and transfer the sample to a microcentrifuge tube.
7. Add 0.4ml of chloroform:isoamyl alcohol (24:1) to lyse the phage, vortex for 1 full minute, and
15 centrifuge in a microcentrifuge (12,000 x g) for 5 minutes. This step removes excess PEG.
8. Transfer the upper, aqueous phase (containing phagemid DNA) to a fresh tube, leaving the interface behind. Add 0.4ml of TE-saturated
20 phenol:chloroform to the aqueous phase, vortex for 1 full minute, and centrifuge as in step 7.
9. Transfer the upper, aqueous phase to a fresh tube and repeat the phenol extraction as in step 8. If necessary, repeat this extraction several times

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until there is no visible material at the interface.

10. Transfer the upper, aqueous phase to a fresh tube and add 0.5 volume (200 μ l) of 7.5M ammonium acetate plus 2 volumes (1.2ml) of ethanol. Mix and leave at -20°C for 30 minutes to precipitate the phagemid DNA.
11. Centrifuge at 12,000 x g for 5 minutes, remove the supernatant, carefully rinse the pellet with 70% ethanol, and centrifuge again for 2 minutes. Drain the tube and dry the pellet under vacuum. The pellet may be difficult to see.
12. Resuspend the DNA in 20 μ l of H₂O. The amount of DNA present can be estimated by agarose gel electrophoresis of a 2 μ l sample.

The mutagenesis reaction involves annealing of the ampicillin repair oligonucleotide and the mutagenic oligonucleotide to the ssDNA template, followed by the synthesis of the mutant strand with T4 DNA polymerase.

20 The heteroduplex DNA is then transformed into the repair minus E. coli strain DMH71-18 mutS or other suitable strain. Mutants are selected by overnight growth in the presence of ampicillin. Plasmid DNA is the isolated and transformed into the JM109 strain, or

25 other suitable strain. Mutant, ampicillin resistant

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colonies may be screened by direct sequencing of the plasmid DNA.

A. ANNEALING REACTION AND MUTANT STRAND SYNTHESIS

The amount of oligonucleotide required in this reaction may vary depending on the size and amount of the single-stranded DNA template. The ampicillin repair oligonucleotide (27 bases long) should be used at a 5:1 oligo:template ratio and the mutagenic oligonucleotide should be used at a 25:1 oligo:template ratio. A typical reaction may contain approximately 100ng (0.05 pmol) of ssDNA.

PROTOCOL

1. Prepare the mutagenesis or control annealing reactions as described below.

15 MUTAGENESIS ANNEALING REACTION

| | |
|--|----------------------|
| Recombinant pSELECT™-1 ssDNA | 0.05pmol |
| Ampicillin repair oligonucleotide (2.2ng/μl) | 1μ (0.25pmol) |
| Mutagenic oligonucleotide, phosphorylated (see Table 1) | 1.25 pmol |
| 10X Annealing buffer | 2μl |
| Sterile H ₂ O | to final volume 20μl |

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CONTROL ANNEALING REACTION

| | | |
|---|-----------------------------------|----------------------------|
| | pSELECT™-Control ssDNA | 100ng (0.05pmol) |
| | Ampicillin repair oligonucleotide | |
| | (2.2ng/ μ l) | 1 μ l (0.25pmol) |
| 5 | lacZ control oligonucleotide | |
| | (10.8ng/ μ l) | 1 μ l (1.25pmol) |
| | 10X Annealing buffer | 2 μ l |
| | Sterile H ₂ O | to final volume 20 μ l |

2. Heat the annealing reaction to 70°C for 5 minutes
10 and allow it to cool slowly to room temperature
(15-20 minutes).

3. Place the annealing reaction on ice and add the
following:

| | | |
|----|----------------------------------|----------------------------|
| | 10X Synthesis buffer | 3 μ l |
| 15 | T4 DNA polymerase (10u/ μ l) | 1 μ l |
| | T4 DNA ligase (2u/ μ l) | 1 μ l |
| | Sterile H ₂ O | 5 μ l |
| | | to final volume 20 μ l |

4. Incubate the reaction at 37°C for 90 minutes to
20 perform mutant strand synthesis and ligation.

TABLE 1. AMOUNT OF MUTAGENIC OLIGONUCLEOTIDE NEEDED TO EQUAL 1.25 PMOL.

| Primer Length | ng of Primer Equal to 1.25pmol |
|---------------|--------------------------------|
| 17mer | 7.0ng |
| 5 20mer | 8.3ng |
| 23mer | 9.5ng |
| 26mer | 10.8ng |
| 29mer | 12.0ng |

B. TRANSFORMATION INTO BMH 71-18 MUTS PROTOCOL

- 10 1. Add 3 μ l of DMSO to 200 μ l of BMH71-18 mut S competent cells, mix briefly, and then add the entire synthesis reaction from step A.4.
2. Let the cells sit on ice for 30 minutes.
3. OPTIONAL: For some strains, a heat shock at 42°C
15 for 1-2 minutes after the incubation on ice has been reported to increase transformation efficiency. In our experience, however, a heat shock does not significantly affect the efficiency of transforming BMH71-18 mut S.
- 20 4. Add 4ml of LB medium and incubate at 37°C for 1 hour to allow the cells to recover.

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5. Add ampicillin to a final concentration of 125 μ g/ml and incubate at 37°C for 12-14 hours with shaking.

NOTE: As a control to check the synthesis reaction, 1 5 ml of the culture can be removed after the one hour recovery step, spun down, resuspended in 50 μ l of LB medium, and plated on LB plates containing 125 μ g/ml ampicillin. This is a check for the presence of ampicillin resistant transformants; a second round of 10 transformation is necessary before screening for mutants.

C. PLASMID MINI-PREP PROCEDURE

This procedure is used to isolate pSELECT-1 or pSELECT-Control plasmid DNA from the overnight culture 15 of BMH 71-18 mut S (step B.5, above). A yield of 1-3 μ g of plasmid DNA may be expected.

PROTOCOL

1. Place 1.5ml of the overnight culture into a microcentrifuge tube and centrifuge at 12, 000 x g 20 for 1 minute. The remainder of the overnight culture can be stored at 4°C.
2. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.
3. Resuspend the pellet by vortexing in 100 μ l of ice- 25 cold miniprep lysis buffer (Promega).

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4. Incubate for 5 minutes at room temperature.
5. Add 200 μ l of a freshly prepared solution containing 0.2N NaOH, 1% SDS. Mix by inversion. DO NOT VORTEX. Incubate for 5 minutes on ice.
- 5 6. Add 150 μ l of ice-cold potassium acetate solution, pH 4.8 (Promega). Mix by inversion or gentle vortexing for 10 seconds. Incubate for 5 minutes on ice.
7. Centrifuge at 12,000 x g for 5 minutes.
- 10 8. Transfer the supernatant to a fresh tube, avoiding the white precipitate.
9. Add 1 volume of TE-saturated phenol/chloroform (Promega). Vortex for 1 minute and centrifuge at 12,000 x g for 5 minutes.
- 15 10. Transfer the upper, aqueous phase to a fresh tube and add 1 volume of chloroform:isoamyl alcohol)24:1). Vortex for 1 minute and centrifuge as in step 9.
11. Transfer the upper, aqueous phase to a fresh tube
20 and add 2.5 volumes of 100% ethanol. Mix and allow to precipitate 5 minutes on dry ice.

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12. Centrifuge at 12,000 x g for 5 minutes. Rinse the pellet with 70% ethanol (prechilled) and dry the pellet under vacuum.
13. Dissolve the pellet in 50 μ l of sterile deionized water. Add 0.5 μ l of 100 μ g/ml DNase-free RNase A (Promega) and incubate for 5 minutes at room temperature.
14. The yield of plasmid DNA can be determined by electrophoresis on an agarose gel.

10 D. TRANSFORMATION INTO JM109 HOST CELLS
PROTOCOL

1. Add 3 μ l of DMSO to 200 μ l of JM109 competent cells, mix briefly, and add 0.05-0.10 μ g of plasmid DNA from step C.14. Other suitable host cells may be used.
2. Let the cells sit on ice for 30 minutes.
3. OPTIONAL: A heat shock may be performed at this step.
4. Add 2ml of LB medium and incubate at 37°C for 1 hour to allow the cells to recover.
5. Divide the culture into two microcentrifuge tubes and spin for 1 minute in a microcentrifuge.

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6. Pour off the supernatant and resuspended the cells in each tube in 50 μ l of LB medium.
7. Plate the cells in each tube on an LB plate containing 125 μ g/ml ampicillin and incubate at 37°C for 12-14 hours.

E. ANALYSIS OF TRANSFORMANTS

The Altered Sites mutagenesis procedure generally produces greater than 50% mutants, so colonies may be screened by direct sequencing. A good strategy is to pick 10 colonies and start by sequencing 4 of these. If the mutation is located within 200-300 bases of either end of the DNA insert, the SP6 or T7 sequencing primers may be used for convenient priming of the sequencing reactions.

15 EXAMPLE 2

CELL CULTURE AND TRANSFECTION

COS-7 cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (GIBCO). Transient expression of cDNAs was performed using a DEAE-Dextran protocol modified by 0.1mM chloroquine treatment (Sussman, D.J. & Milman, *Mol. Cell Biol.* 4:1641-1645 (1984); Ausubel, F.M., et al., "Current Protocols in Molecular Biology" pp.921-926, John Wiley and Son, New York, (1989)). 3 days before the transfection, COS-7 cells were plated at 2×10^5 /10-cm tissue culture dish. 4 μ g DNA were used in

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each transfection. Medium was collected 3 days after transfection and assayed for erythropoietin activity and protein.

EXAMPLE 3

5 IMMUNOPRECIPITATION OF ERYTHROPOIETIN

Wildtype and mutant erythropoietin contained in supernatant medium from COS cell transfections were diluted one- to four-fold with Dulbecco's modified Eagle medium containing 10% fetal bovine serum. After
10 one hour incubation at 37 degrees C with a monoclonal anti-peptide antibody to erythropoietin directed against amino acids 1-26 or 99-129, an equal volume of Omnisorb (Calbiochem) was added to the samples and the suspension was incubated for one hour at 4 degrees C.
15 The Omnisorb was pelleted by centrifugation at 4000 rpm for 30 seconds. The erythropoietin remaining in the supernatant which was not bound by the monoclonal antibody was measured by radioimmunoassay. The amount of erythropoietin bound by antibody (as a percent) was
20 calculated by subtracting the amount in the supernatant from 100%, the starting concentration.

EXAMPLE 4

MODIFIED ERYTHROPOIETIN VARIANT PROTEINS PRODUCED BY
ALTERING NONCODING REGIONS OF THE GENE

25 Typically, variants of recombinant proteins are made by deleting, adding or substituting nucleotides within the coding of the gene. However, it is also possible to make variants of recombinant proteins by

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altering the noncoding regions of genes, i.e., the 5' and 3' untranslated regions (UTR). Modifications in the UTR of a gene, especially in the 5' sequence as well as in the first intron, influence the regulation of translation; and, thus, the expression of proteins (Schultz, D.E., et al., *J. Virol.* 70:1041-1049, 1996; Kozak, M., *J. Mol. Biol.* 235:95-110, 1994; Bettany, A.J., et al., *J. Biol. Chem.* 267:16531-16537, 1992; Kozak, M., *J. Biol. Chem.* 266:19867-19870, 1991).

10 Alterations in the non-coding sequences of the erythropoietin gene can result in different mRNA secondary structure (e.g., free energy of the loop and base pairs), translation efficiency; and subsequently, the expression, secretion and biological activity of
15 the erythropoietin. Therefore, different forms of modified erythropoietin proteins can be manufactured as a result of modifications in regions which flank either the 5' or 3' side of the coding region of the erythropoietin gene.

20 Figure 7 is a schematic representation of changes in mRNA structure and ultimately protein structure and function that can result when an alteration(s) is made in the 5' and/or 3' UTR of the erythropoietin gene. Variations in the modified erythropoietin proteins can
25 be produced as, for example, different restriction enzyme generated fragments of genomic sequences and/or specific nucleotide substitutions and mutations in the 5' and/or 3' UTR of the erythropoietin coding sequence. Oligonucleotide-directed site-specific mutagenesis

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procedures as described herein can be employed to provide the modified erythropoietin variant proteins.

Alterations in the noncoding regions of the erythropoietin gene can affect mRNA stability, rates of translation, expression from host cells, protein processing, export from rough endoplasmic reticulum, extent and pattern of glycosylation, secretion dynamics and rates of export from the cell. For example, varied glycosylation patterns can result, which, for erythropoietin, are of great importance for biological activity (Yamaguchi, K., et al., *J. Biol. Chem.* 266:20434-20439, 1991). The resulting proteins can represent chemically, structurally and biologically distinct forms of the modified erythropoietin variant proteins.

The nucleotide sequences of the modified erythropoietin variant proteins can be confirmed by DNA sequencing using standard experimental procedures. Distinctive versions of genomic erythropoietin can be produced by mutations in the 5' and 3' UTR and detected by Southern blotting. Likewise, different mRNAs can be identified by Northern blotting. Differences in hybridization conditions, i.e., high or low stringencies, will be an index of the diversity of the DNA and mRNA. It is possible that different genomic sequences may require different promoters (e.g., mouse metallothionein or 3-phosphoglycerate), vectors (e.g., bovine papilloma virus), and/or host cells (e.g., CHO, BHK-21 or C127 cells) to adequately express the modified erythropoietin variant proteins. The

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technical methods which can be employed for the above mentioned experimental strategies are familiar to those of skill in the art. For example, detailed protocols can be found in Sambrook, et al., "Molecular Cloning: 5 A Laboratory Manual," (1989) and Ausubel, et al., "Current Protocols in Molecular Biology, "(1995); Powell, J.S., et al., Proc. Natl. Acad. Sci. USA 83:6465-6469, 1986; Sytkowski and Grodberg, (U.S. Patent No. 5,614,184); Sytkowski (U.S. Patent No. 10 5,580,853); and Powell (U.S. Patent No. 5,688,679); the teachings of which are herein incorporated by reference in their entirety.

Mutations in the 5' and/or 3' UTR of the erythropoietin gene can result in altered RNA 15 structure, total free energy, stability and/or rates and efficiency of translation (Schultz, D.E., et al., J. Virol. 70:1041-1049, 1996; Kozak, M., J. Mol. Biol. 235:95-110, 1994; Bettany, A.J., et al., J. Biol. Chem. 267:16531-16537, 1992; Kozak, M., J. Biol. Chem. 20 266:19867-19870, 1991; Purvis, I.J., et al., Nucleic Acids Res. 15: 7951-62, 1987). The secondary structure of mRNAs play an important role in the initiation and efficiency of translation and, thus, in protein synthesis.

25 Computer modeling using the PC/Gene® RNAFOLD program (IntelliGenetics, Inc.) is used to predict differences in RNA secondary structure, specifically the total free energy, following deletion in the 5' or 3' UTR of, for example, the erythropoietin gene 30 (Figures 9-10). The program utilizes an algorithm

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which calculates the energies of the secondary structure of RNA. It automatically transcribes any DNA sequence into a single stranded RNA sequence. Since the mRNA is single stranded, it can fold back upon 5 itself due to the complementarity of bases resulting in various "loops". Energy must be released to form a base-paired or looped structure and the stability of the resulting secondary structure is determined by the amount of energy released. Therefore, if alternative 10 structures have a free energy of formation of -50 kcal/mol and -100 kcal/mol, the latter structure is intrinsically more likely to be formed.

For example, the free energy for RNA secondary structure for nucleotides 401-624 in the 5' UTR of the 15 erythropoietin gene is predicted to be -161.0 kcal/mol (SEQ ID NO:24). A 50 nucleotide deletion spanning nucleotides 501-550 results in a total free energy of -127.2 kcal/mol (SEQ ID NO:25), whereas a 50 nucleotide deletion at nucleotides 551-600 (SEQ ID NO:26) results 20 in an RNA structure with -118.9 kcal/mol of free energy indicating the importance of the size of the deletion and location in ultimately defining mRNA secondary structure. Larger deletions, in different portions of the 401-624 region of the 5' UTR, yield RNA structures 25 with varying predicted energy states (SEQ ID NOS:27-29). These results are summarized in Table 2.

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TABLE 2: SEQUENCE VARIATION IN 5' UTR-
EFFECT ON mRNA FREE ENERGY

| Sequence | SEQ ID NO: | Nucleotide Length (bp) | Region of Deletion | Number of Nucleotides Deleted (bp) | Free Energy (kal/mol) |
|----------|------------|------------------------|--------------------|------------------------------------|-----------------------|
| Native | 24 | 224 | --- | --- | -161.0 |
| 5'a | 25 | 174 | 501-550 | 50 | -127.2 |
| 5'b | 26 | 174 | 551-600 | 50 | -118.9 |
| 5'c | 27 | 124 | 401-550 | 100 | -94.1 |
| 5'd | 28 | 74 | 401-550 | 150 | -52.3 |
| 5'e | 29 | 34 | 401-590 | 190 | -11.3 |

10 Likewise, for example, the free energy of RNA
secondary structure for nucleotides 2773-2972 in the
3' UTR of the erythropoietin gene is predicted to
be -81.4 kcal/mol (SEQ ID NO:30). A 50 nucleotide
deletion spanning nucleotides 2923-2972 (SEQ ID
15 NO:31) results in a total free energy of -53.5
kcal/mol, whereas a 100 nucleotide deletion at
nucleotides 2873-2972 (SEQ ID NO:32) results in an
RNA structure with -33.3 kcal/mol of free energy.
Larger deletions, in different portions of the 2773-
20 2973 region of the 3' UTR, yield RNA structures with
varying predicted energy states (SEQ ID NOS:33 and
34). These results are summarized in Table 3.

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TABLE 3: SEQUENCE VARIATION IN 3' UTR-
EFFECT ON mRNA FREE ENERGY

| Sequence | SEQ ID NO: | Nucleotide Length (bp) | Region of Deletion | Number of Nucleotides Deleted (bp) | Free Energy (kal/mol) |
|----------|------------|------------------------|--------------------|------------------------------------|-----------------------|
| Native | 30 | 200 | --- | --- | -81.4 |
| 3'a | 31 | 150 | 2923-2972 | 50 | -53.5 |
| 3'b | 32 | 100 | 2873-2972 | 100 | -33.3 |
| 3'c | 33 | 50 | 2823-2972 | 150 | -12.5 |
| 3'd | 34 | 100 | 2801-2900 | 100 | -36.6 |

The secondary structure of mRNA affects the rates of translation of the corresponding coding regions (Kikinis, Z., et al., *Nucleic Acids Res.* 23: 4190-4195, 1995; Kozak, M., *Mamm. Genome* 7: 563-574, 1996; Bettany, A.J., et al., *J. Biol. Chem.* 267: 16531-16537, 1992; Kozak, M., *J. Mol. Biol.* 235: 95-110, 1994). Secondary structure loops in the mRNA must be unwound to facilitate ribosome attachment and proper protein assembly (Alberts, B., et al., "Molecular Biology of the Cell", 3rd ed., Garland Publishing, Inc., New York, NY, pp. 223-290, 1994).

The nascent polypeptide chains can interact with chaperon proteins, for example, BiP, in unique ways which can affect the proper folding of the polypeptide chain and influence passage of the protein through the endoplasmic reticulum thereby altering glycosylation of the resulting protein.

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Recent data suggest that BiP-like proteins not only bind improperly folded proteins but also may assist in the appropriate protein folding and facilitate the membrane translocation and glycosylation of secretory proteins. (Knittler, M.R., et al., *EMBO J.*, 11:1573-1581, (1992); Sanders, S.L., et al., *Cell*, 69:353-365, (1992)). Alterations in glycosylation patterns can influence the secretion and, in the case of erythropoietin, drastically alter biological activity (Yamaguchi, K., et al., *J. Biol. Chem.*, 266:20434-20439, 1991).

The three dimensional structure of erythropoietin is significantly influenced by the protein backbone and the oligosaccharide chains. Alterations in the carbohydrate composition (e.g., the number of N- or O-linked oligosaccharide residues and/or type of sugar moieties) can lead to different biological properties of the modified erythropoietin variant proteins and, thus, different therapeutic effects. Therefore, an alteration in the 5' or 3' UTR can affect mRNA secondary structure, which in turn can influence the rate of expression and post-translational modifications such as glycosylation. The proper glycosylation of erythropoietin is of paramount importance to proper folding and secretion of the mature product and, hence, its biological and pharmacological properties.

Indices of intrinsic structural variations in the modified erythropoietin proteins can be

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manifested in differences in the three-dimensional structure of the protein backbone and the extent and pattern of carbohydrate chains. For example, circular dichroism (CD) spectra and thermal stability for the resulting erythropoietin mutant proteins can be performed to determine the content of alpha helix, beta sheet, beta turn and random coil for different glycoproteins. The structure of the oligosaccharide chains can be determined, for example, using enzymatic and chemical deglycosylation, gas chromatography, methylation analyses, fast-atom-bombardment mass spectrometry as well as one-and two-dimensional ¹H-NMR spectrometry. The methods to perform the above mentioned analyses are routine to one of ordinary skill in the art and are delineated in detail in several references including for example, Ausubel, F.M., et al., "Current Protocols in Molecular Biology" (1995); Nimtz, M., et al. *Eur. J. Biochem.* 213: 39-56, 1993; and Nimtz, M., et al., *FEBS* 365: 203-208, 1995, the teachings of which are herein incorporated by reference in their entirety.

In addition, assessment of the structural differences in the modified erythropoietin variant proteins can be evaluated using immunoprecipitation with erythropoietin-specific monoclonal antibodies and heat denaturation curves. Experimental techniques to measure these properties of erythropoietin are described in Sytkowski and Grodberg (U.S. Patent No. 5,614,184); Sytkowski

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(U.S. Patent No. 5,580,853); and Powell (U.S. Patent No. 5,688,679); the teachings of which are herein incorporated by reference in their entirety.

EXAMPLE 5

5 EVALUATION OF BIOLOGICAL ACTIVITY OF MODIFIED ERYTHROPOIETIN VARIANT PROTEINS

The biological activity of the modified erythropoietin variant proteins is determined using *in vitro* and *in vivo* assays.

10 The modified erythropoietin variant proteins can be preferably purified substantially prior to use, particularly where the protein could be employed as an *in vivo* therapeutic, although the degree of purity is not necessarily critical where
15 the molecule is to be used *in vitro*. The modified erythropoietin variant proteins can be isolated to about 50% purity (by weight), more preferably to about 80% by weight or about 95% by weight. It is most preferred to utilize a protein which is
20 essentially pure (e.g., about 99% by weight or to homogeneity) for *in vitro* and *in vivo* assays as well as *in vivo* therapeutics.

For example, the modified erythropoietin variant proteins, which are prepared according to
25 the methods discussed in the Examples, can be screened for *in vitro* and *in vivo* activity prior to use in therapeutic settings. The *in vitro* assay measures the effect of erythropoietin variant proteins on erythropoiesis in intact mouse spleen

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cells according to the procedure of Krystal, G.,
Exp. Hematol. 11:649-660 (1983). To screen the
various modified erythropoietin variant proteins for
activity, for example, *in vitro* or *in vivo*, the
5 proteins (or mixtures of the modified erythropoietin
variant proteins) can be evaluated for the extent of
erythropoiesis or receptor binding. Tests to
determine biological activity are well-known to
those of skill in the art. For example, the
10 biological activity of erythropoietin can be
measured as described in Sytkowski and Grodberg
(U.S. Patent No. 5,614,184); Sytkowski (U.S. Patent
Nos 5,580,853); Sytkowski, U.S. patent application
"Modified Polypeptides with Altered Biological
15 Activity", filed February 3, 1998; and Powell (U.S.
Patent No. 5,688,679); the teachings of which are
herein incorporated by reference in their entirety.

EQUIVALENTS

While this invention has been particularly
20 shown and described with references to preferred
embodiments thereof, it will be understood by those
skilled in the art that various changes in form and
details may be made therein without departing from
the spirit and scope of the invention as defined by
25 the appended claims. Those skilled in the art will
recognize or be able to ascertain using no more than
routine experimentation, many equivalents to the
specific embodiments of the invention described

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specifically herein. Such equivalents are intended to be encompassed in the scope of the claims.

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CLAIMS

What is claimed is:

1. An isolated nucleic acid encoding erythropoietin wherein the nucleic acid has one
5 or more mutations in a noncoding region, and wherein the erythropoietin has altered biological activity.
2. The nucleic acid of Claim 1, wherein the mutation is in the 5' noncoding region.
- 10 3. The nucleic acid of Claim 2 wherein the nucleic acid comprises a nucleic acid selected from the group consisting of SEQ ID NO:25; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO: 28; and SEQ ID NO: 29.
4. A composition comprising an erythropoietin
15 protein of Claim 2 and a pharmaceutically acceptable carrier.
5. A method of treating or preventing anemia in a mammal comprising administering to the mammal a therapeutically effective amount of an
20 erythropoietin protein encoded by the nucleic acid of Claim 2.
6. The nucleic acid of Claim 1, wherein the mutation is in the 3' noncoding region.

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7. The nucleic acid of Claim 6 wherein the nucleic acid comprises a nucleic acid selected from the group consisting of SEQ ID NO: 31; SEQ ID NO: 32; SEQ ID NO: 33; and SEQ ID NO: 34.
- 5 8. A composition comprising an erythropoietin protein of Claim 6 and a pharmaceutically acceptable carrier.
9. A method of treating or preventing anemia in a mammal comprising administering to the mammal a
10 therapeutically effective amount of an erythropoietin protein encoded by the nucleic acid of Claim 6.
10. The nucleic acid of Claim 1 wherein the
15 mutation is in both the 5' and 3' noncoding region.
11. The nucleic acid of Claim 10 wherein the nucleic acid comprises a nucleic acid selected from the group consisting of SEQ ID NO:25; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO: 28; SEQ ID
20 NO: 29; SEQ ID NO: 31; SEQ ID NO: 32; SEQ ID NO: 33; and SEQ ID NO: 34.

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12. A composition comprising an erythropoietin protein of Claim 10 and a pharmaceutically acceptable carrier.
13. A method of treating or preventing anemia in a mammal comprising administering to the mammal a therapeutically effective amount of an erythropoietin protein encoded by the nucleic acid of Claim 10.
14. The nucleic acid sequence of Claim 1 further comprising one or more mutations in the coding region encoding erythropoietin having an amino acid residue which differs from the amino acid residue present in the corresponding position in wildtype erythropoietin, the amino acid residue of wildtype erythropoietin selected from the group consisting of amino acid 101, amino acid residue 103, amino acid residue 104, amino acid residue 105 and amino acid residue 108.
15. A mutant erythropoietin protein according to Claim 14, wherein the amino acid residue at the position 101 is alanine.
16. A mutant erythropoietin protein according to Claim 14, wherein the amino acid

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residue at the position 103 is selected from the group consisting of aspartate, alanine, glutamate, histidine and lysine.

17. A mutant erythropoietin protein according
5 to Claim 14, wherein the amino acid residue at the position 104 is alanine.
18. A mutant erythropoietin protein according to Claim 14, wherein the amino acid residue at the position 105 is alanine.
- 10 19. A mutant erythropoietin protein according to Claim 14, wherein the amino acid residue at the position 108 is alanine.

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ALANINE SCANNING MUTAGENESIS OF EPO 100-109

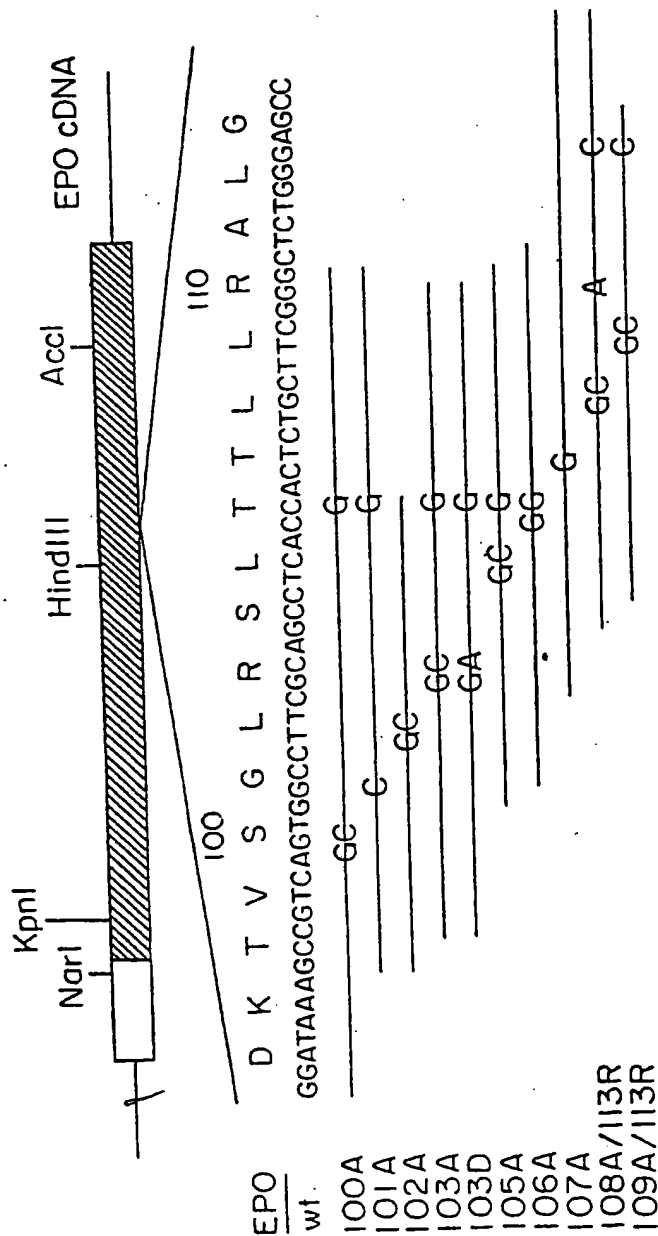


FIG. 1

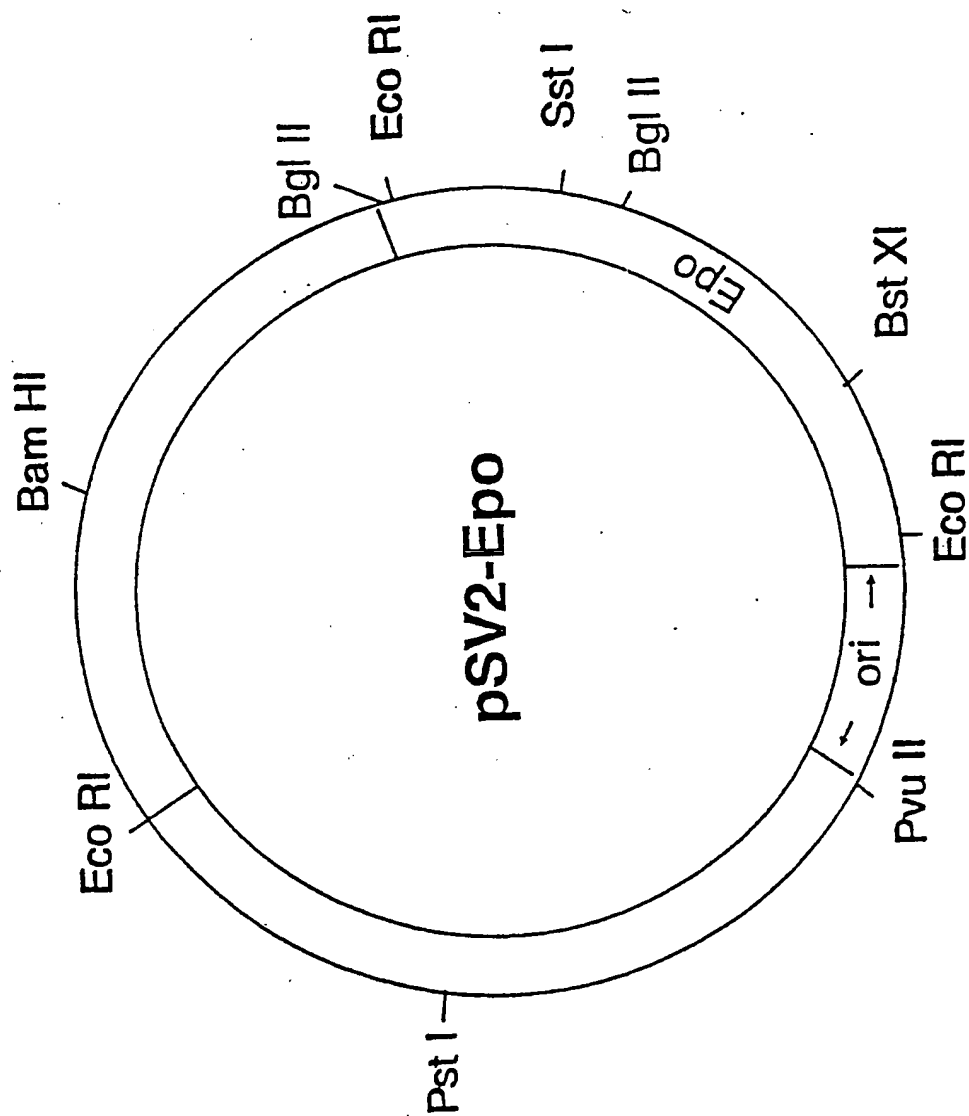


FIG. 2

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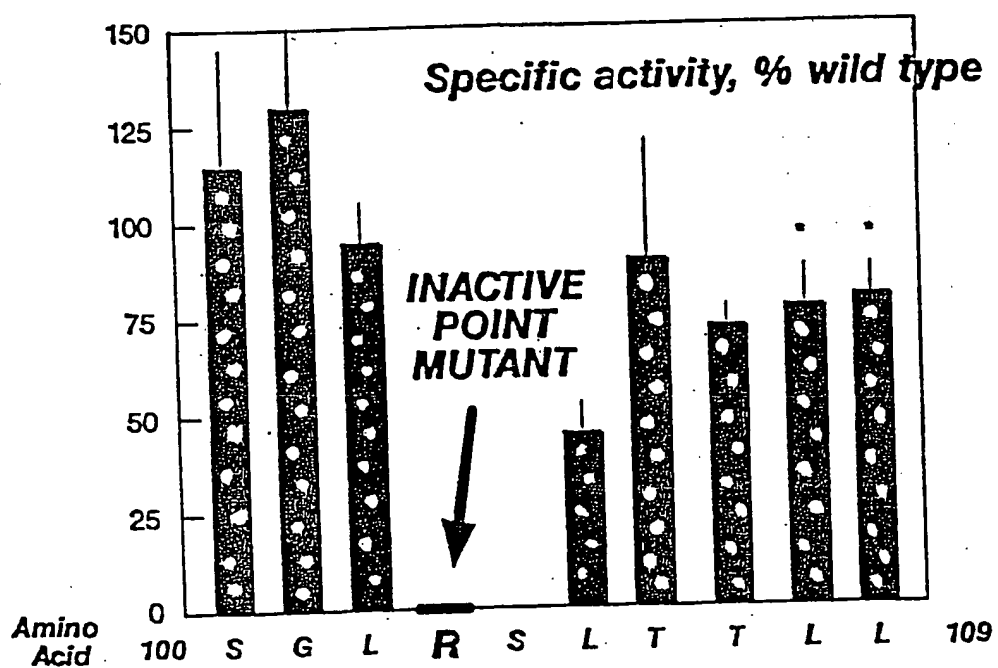
ARGININE 103 IS ESSENTIAL FOR EPO'S ACTIVITY

FIG. 3

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**MoAbs TO AMINO ACIDS 1-26 AND 99-129
PROBE THE STRUCTURE OF EPO**

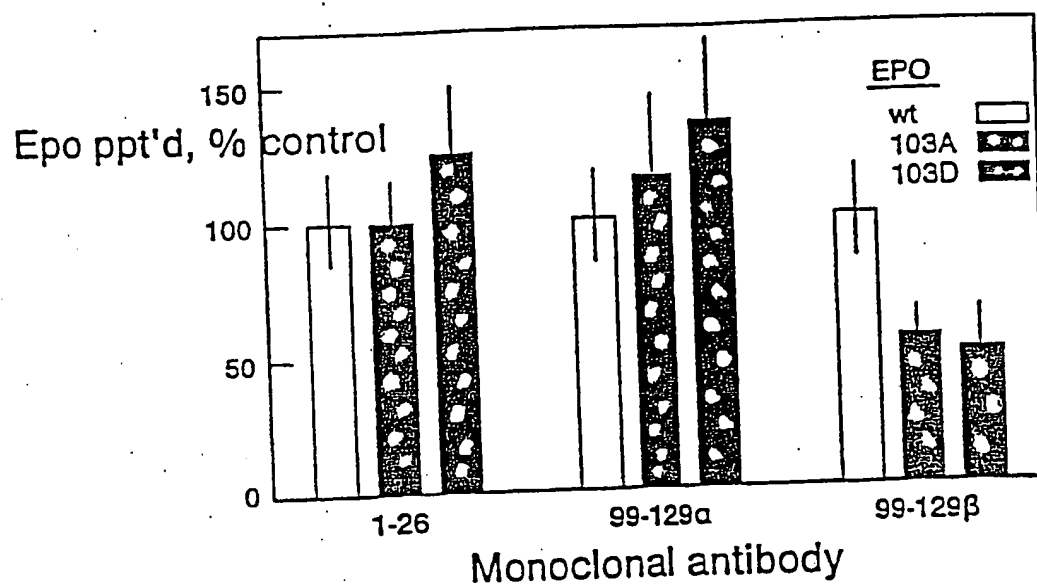


FIG. 4

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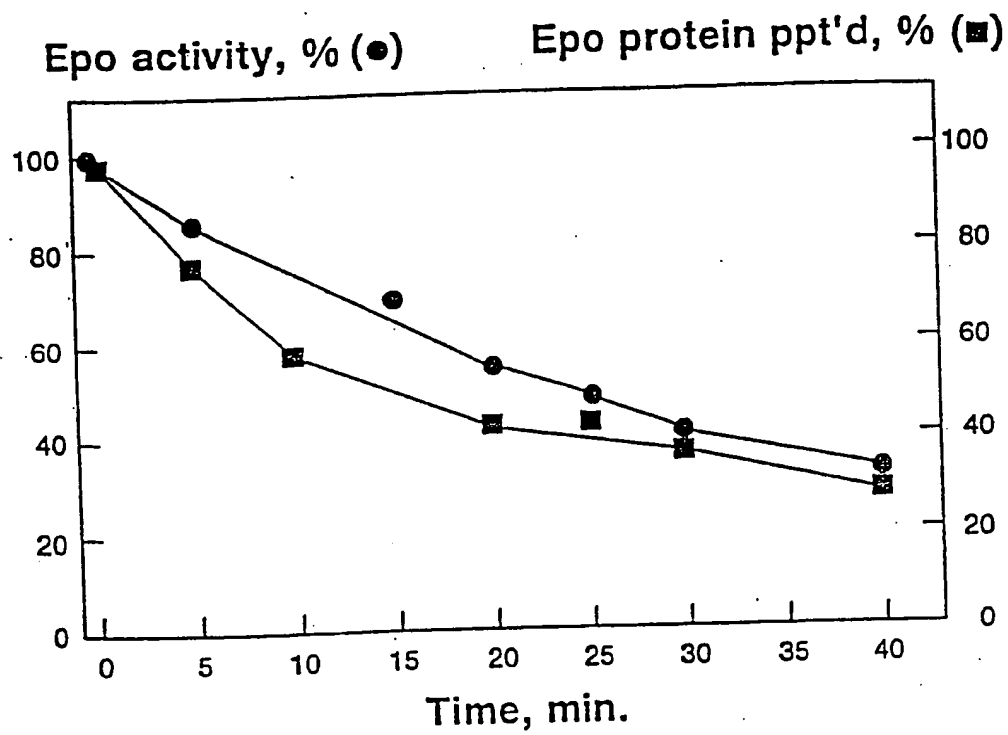


FIG. 5

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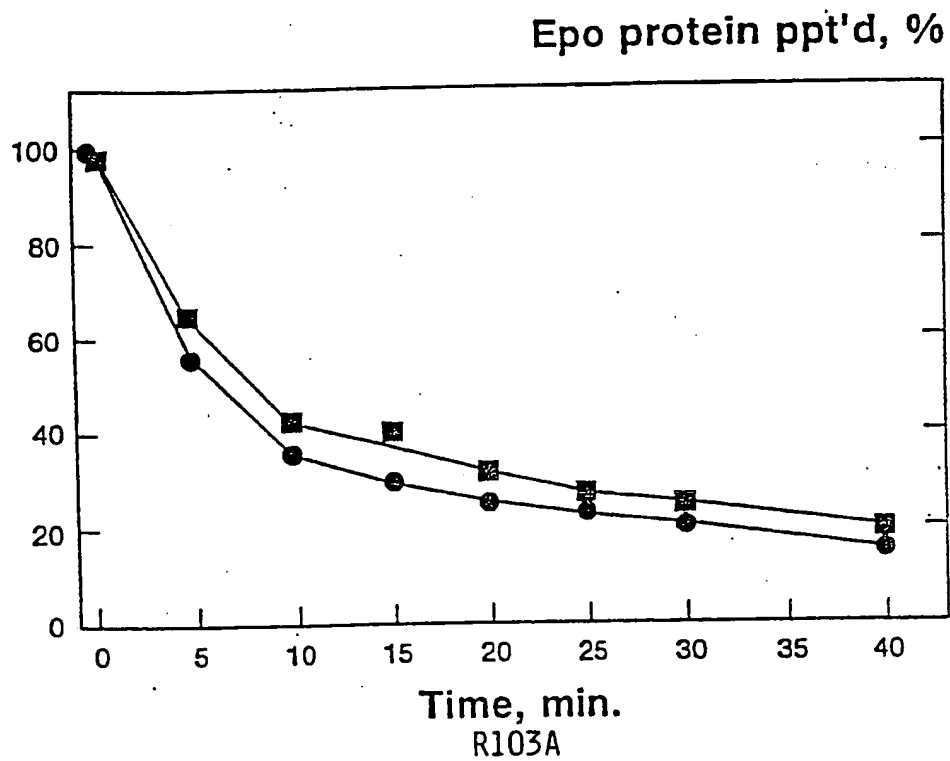


FIG. 6A

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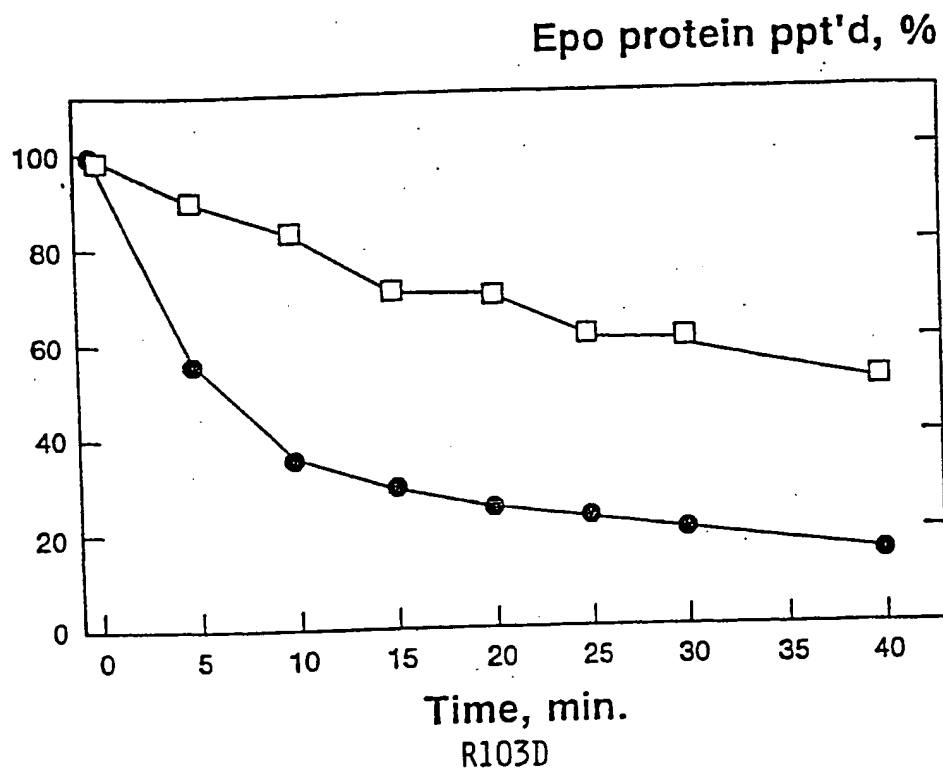
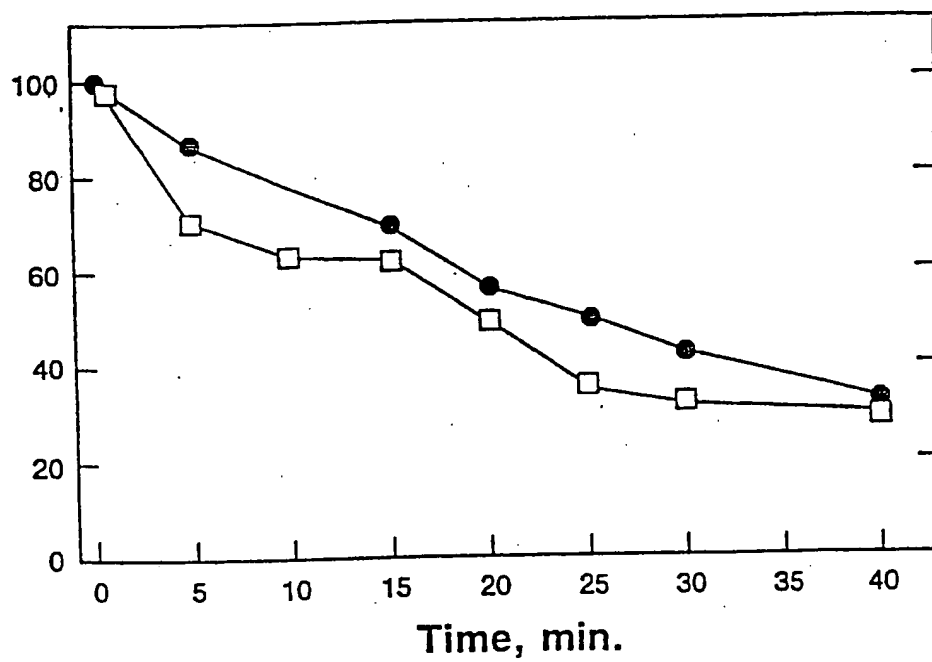


FIG. 6B

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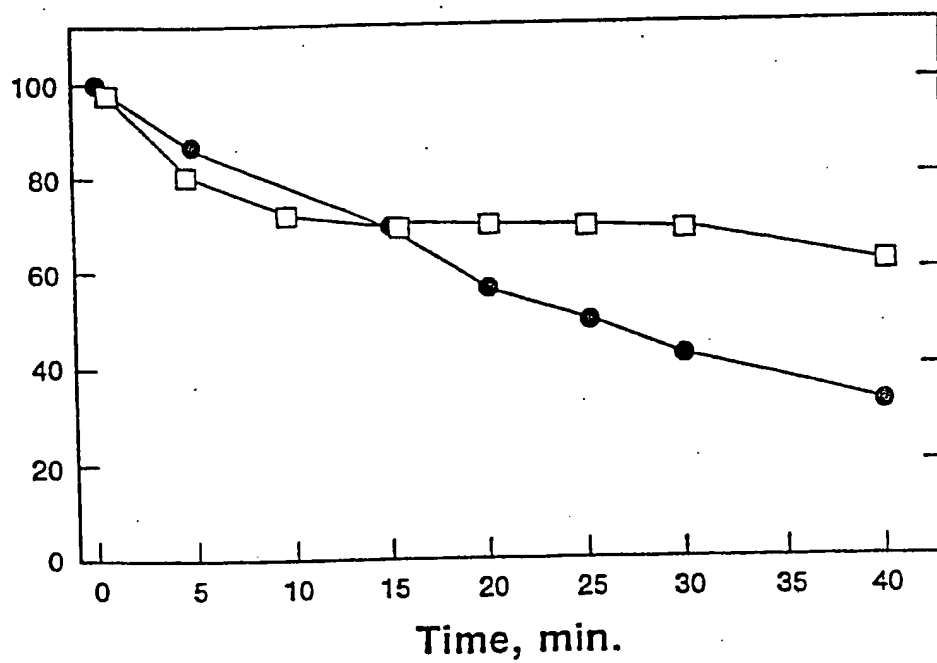


Time, min.

R103K

FIG. 6C

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R103E

FIG. 6D

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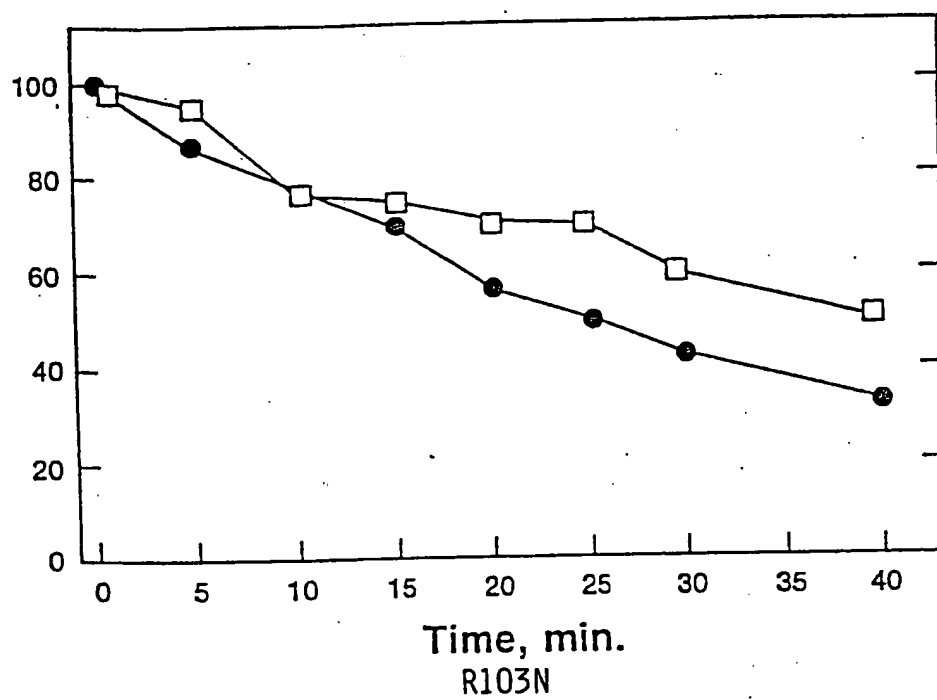


FIG. 6E

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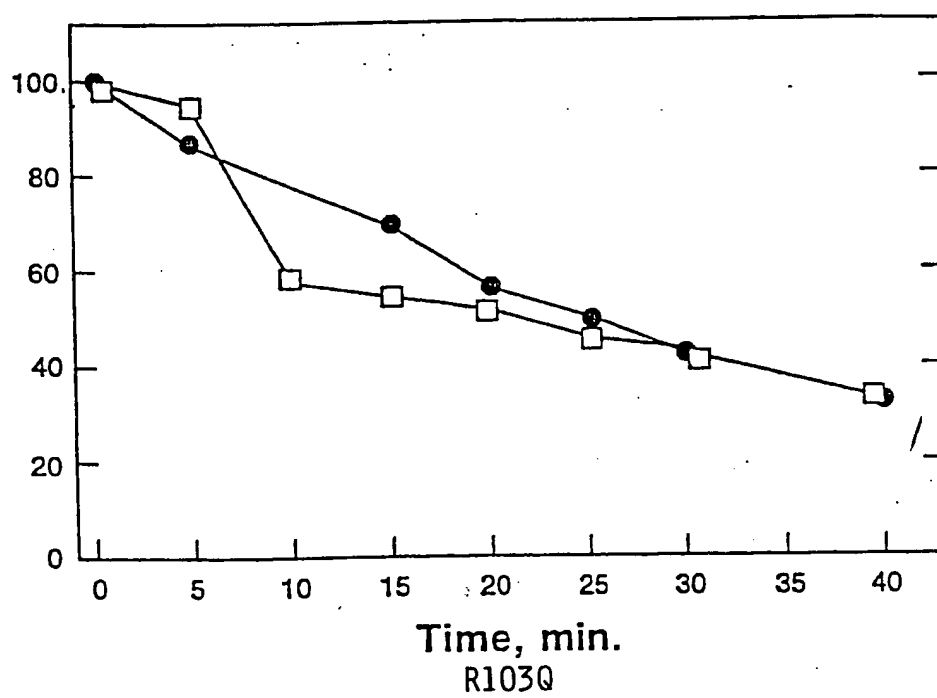
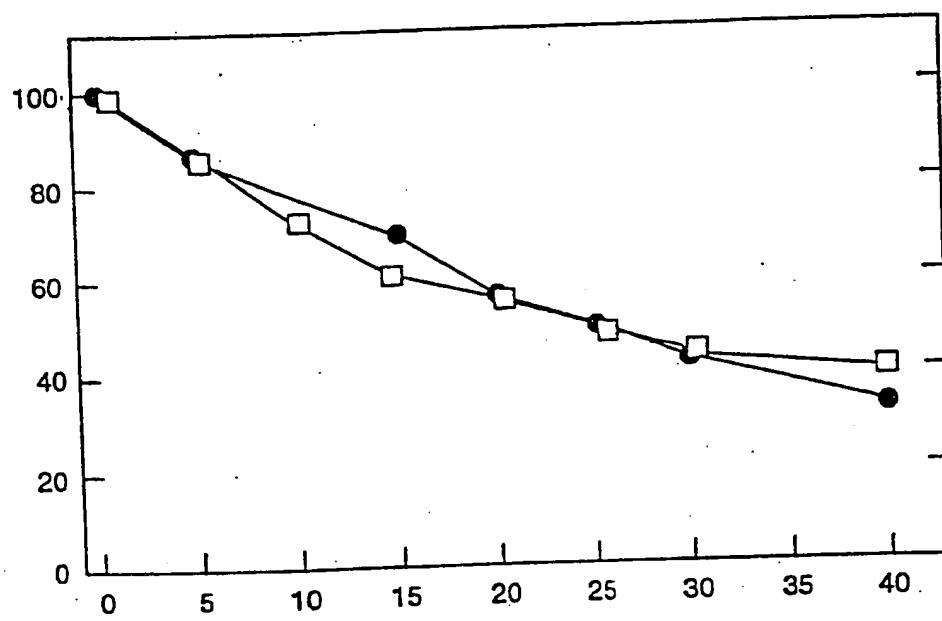


FIG. 6F

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Time, min.

R103H

FIG. 6G

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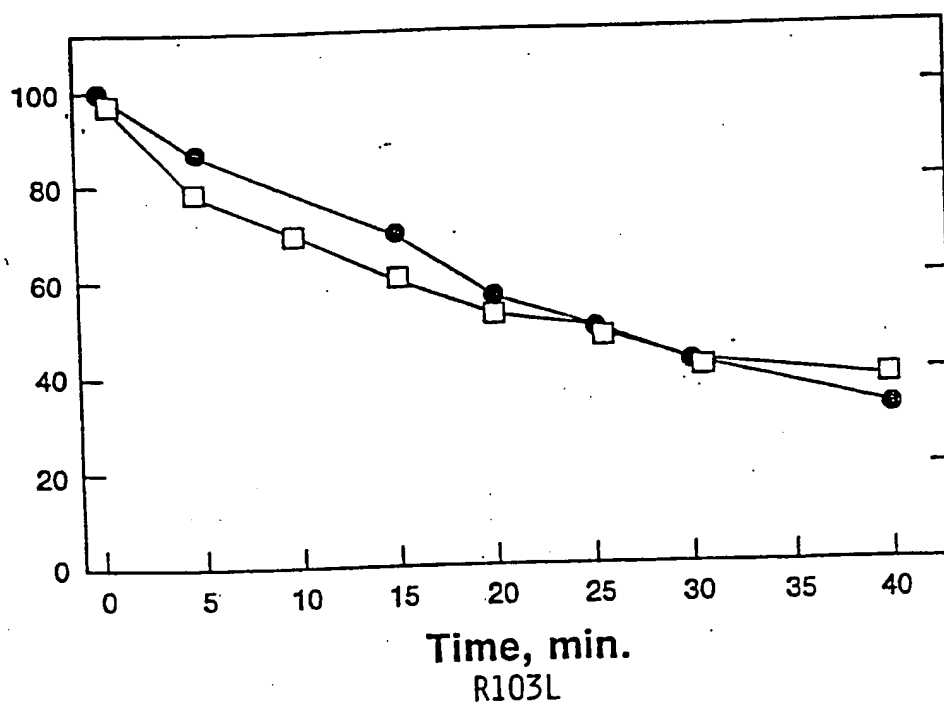


FIG. 6H

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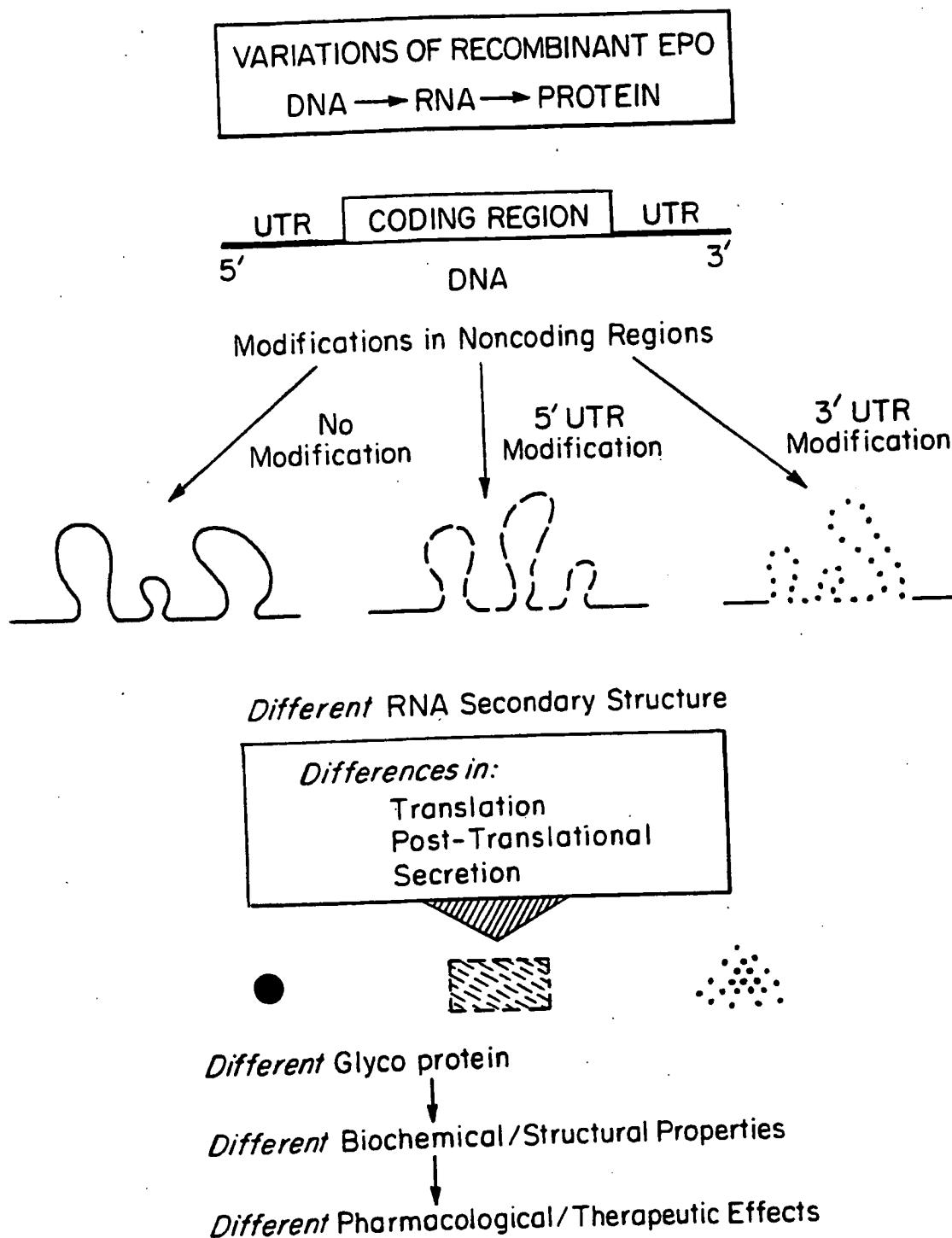


FIG. 7

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HUMAN ERYTHROPOIETIN GENE

From: HOMO SAPIENS (HUMAN)

EUKARYOTA; ANIMALIA; METAZOA; CHORDATA; VERTEBRATA; MAMMALIA;
THERIA; EUTHERIA; PRIMATES; HAPLORHINI; CATARRHINI; HOMINIDAE.

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1   AAGCTTCTGG GCTTCCAGAC CCAGCTACTT TGCGGAATC AGCAACCCAG GCATCTCTGA
61  GTCTCCGCCC AAGACCGGGA TGCCCCCAG GGGAGGTGTC CGGGAGCCCA GCCTTTCCCA
121 GATAGCACGC TCCGCCAGTC CCAAGGGTGC GCAACCGGCT GCACTCCCCT CCCGCGACCC
181 AGGGCCCGGG AGCAGCCCCC ATGACCACAC CGCAGTCTG CAGCAGCCCC GCTCACGCCC
241 CGGCGAGCCT CAACCCAGGC GTCCTGCCCC TGCTCTGACC CCGGTGGGCC CCTACCCCTG
301 GCGACCCCTC ACGCACACAG CCTCTCCCC ACCCCCACCC GCGCACGCAC ACATGCAGAT
361 AACAGCCCCG ACCCCCGGCC AGAGCCGCAG AGTCCCTGGG CCACCCCGGC CGCTCGCTGC
421 GCTGCGCCGC ACCGCGCTGT CCTCCCGGAG CCGGACCGGG GCCACCGCGC CCGCTCTGCT
481 CCGACACCGC GCCCCCTGGA CAGCCGCCCT CTCCTCTAGG CCCGTGGGGC TGGCCCTGCA
541 CCGCCGAGCT TCCCGGGATG AGGGCCCCCG GTGTGGTCAC CCGGCGCGCC CCAGGTCGCT
601 GAGGGACCCC GGCCAGGCGC GGAGATGGGG GTGCACGGTG AGTACTCGCG GGCTGGGCGC
661 TCCCGCCGCC CGGGTCCCTG TTTGAGCGGG GATTTAGCGC CCCGGCTATT GGCCAGGAGG
721 TGGCTGGGTT CAAGGACCGG CCACTTGTCA AGGACCCCGG AAGGGGGAGG GGGGTGGGGC
781 AGCCTCCACG TGCCAGCGGG GACTTGGGGG AGTCCTTGGG GATGGCAAAA ACCTGACCTG
841 TGAAGGGGAC ACAGTTTGGG GGTGAGGGG AAGAAGGTTT GGGGGTTCTG CTGTGCCAGT
901 GGAGAGGAAG CTGATAAGCT GATAACCTGG GCGCTGGAGC CACCACTTAT CTGCCAGAGG
961 GGAAGCCTCT GTCACACCAG GATTGAAGTT TGGCCGGAGA AGTGGATGCT GGTAGCTGGG
1021 GGTGGGGTGT GCACACGGCA GCAGGATTGA ATGAAGGCCA GGGAGGCAGC ACCTGAGTGC
1081 TTGCATGGTT GGGGACAGGA AGGACGAGCT GGGGCAGAGA CGTGGGGATG AAGGAAGCTG
1141 TCCTTCCACA GCCACCCTTC TCCCTCCCCG CCTGACTCTC AGCCTGGCTA TCTGTTCTAG
1201 AATGTCCTGC CTGGCTGTGG CTTCTCCTGT CCCTGCTGTC GCTCCCTCTG GGCCTCCAG
1261 TCCTGGGCGC CCCACCACGC CTCATCTGTG ACAGCCGAGT CCTGGAGAGG TACCTCTTGG
1321 AGGCCAAGGA GGCCGAGAAT ATCACGGTGA GACCCCTTCC CCAGCACATT CCACAGAACT

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FIGURE 8A

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1381 CACGCTCAGG GCTTCAGGGA ACTCCTCCCA GATCCAGGAA CCTGGCACTT GGTTTGGGGT
1411 GGAGTTGGGA AGCTAGACAC TGCCCCCCTA CATAAGAATA AGTCTGGTGG CCCCAAACCA
1501 TACCTGGAAA CTAGGCAAGG AGCAAAGCCA GCAGATCCTA CGGCCTGTGG GCCAGGGCCA
1561 GAGCCTTCAG GGACCCTTGA CTCCCCGGGC TGTGTGCATT TCAGACGGGC TGTGCTGAAC
1621 ACTGCAGCTT GAATGAGAAT ATCACTGTCC CAGACACCAA AGTTAATTTT TATGCTGGA
1681 AGAGGATGGA GGTGAGTTCC TTTTTTTTTT TTTTTCCTTT CTTTTGGAGA ATCTCATTG
1741 CGAGCCTGAT TTTGGATGAA AGGGAGAATG ATCGGGGGAA AGGTAAATG GAGCAGCAGA
1801 GATGAGGCTG CCTGGGCGCA GAGGCTCACG TCTATAATCC CAGGCTGAGA TGGCCGAGAT
1861 GGGAGAATTG CTTGAGCCCT GGAGTTTCAG ACCAACCTAG GCAGCATAGT GAGATCCCCC
1921 ATCTCTACAA ACATTTAAAA AAATTAGTCA GGTGAAGTGG TGCATGGTGG TAGTCCCAGA
1981 TATTTGGAAG GCTGAGGCGG GAGGATCGCT TGAGCCCAGG AATTTGAGGC TGCAGTGAGC
2041 TGTGATCACA CCACTGCACT CCAGCCTCAG TGACAGAGTG AGGCCCTGTC TCAAAAAAGA
2101 AAAGAAAAAA GAAAAATAAT GAGGGCTGTA TGGAAATACAT TCATTATTCA TTCACTCACT
2161 CACTCACTCA TTCATTCACT CATTCATTCA ACAAGTCTTA TTGCATACCT TCTGTTTGCT
2221 CAGCTTGGTG CTTGGGGCTG CTGAGGGGCA GGAGGGAGAG GGTGACATGG GTCAGCTGAC
2281 TCCCAGAGTC CACTCCCGTG AGGTCGGGCA GCAGGCCGTA GAAGTCTGGC AGGGCCTGGC
2341 CCTGCTGTCC GAAGCTGTCC TGCGGGGCCA GGCCCTGTTG GTCAACTCTT CCCAGCCGTG
2401 GGAGCCCCTG CAGCTGCATG TGGATAAAGC CGTCAGTGGC CTTGCGAGCC TCACCACTCT
2461 GCTTCGGGCT CTGGGAGCCC AGGTGAGTAG GAGCGGACAC TTCTGCTTGC CCTTTCTGTA
2521 AGAAGGGGAG AAGGGTCTTG CTAAGGAGTA CAGGAACTGT CCGTATTCCT TCCCTTTCTG
2581 TGGCACTGCA GCGACCTCCT GTTTTCTCCT TGGCAGAAGG AAGCCATCTC CCCTCCAGAT
2641 GCGGCCTCAG CTGCTCCACT CCGAACAATC ACTGCTGACA CTTTCCGCAA ACTCTTCCGA
2701 GTCTACTCCA ATTTCTCCCG GGGAAAGCTG AAGCTGTACA CAGGGGAGGC CTGCAGGACA
2761 GGGGACAGAT GACCAGGTGT GTCCACCTGG GCATATCCAC CACCTCCCTC ACCAACATTG
2821 CTTGTGCCAC ACCCTCCCCC GCCACTCCTG AACCCCGTCG AGGGGCTCTC AGCTCAGCGC
2881 CAGCCTGTCC CATGGACACT CCAGTGCCAG CAATGACATC TCAGGGGCCA GAGGAACTGT
2941 CCAGAGAGCA ACTCTGAGAT CTAAGGATGT CACAGGGCCA ACTTGAGGGC CCAGAGCAGG

FIGURE 8B

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3001 AAGCATTGAG AGAGCAGCTT TAAACTCAGG GACAGAGCCA TGCTGGGAAG ACGCCTGAGC
3061 TCACTCGGCA CCCTGCAAAA TTTGATGCCA GGACACGCTT TGGAGGCGAT TTACCTGTTT
3121 TCGCACCTAC CATCAGGGAC AGGATGACCT GGAGAACTTA GGTGGCAAGC TGTGACTTCT
3181 CCAGGTCTCA CGGGCATGGG CACTCCCTTG GTGGCAAGAG CCCCCTTGAC ACCGGGGTGG
3241 TGGGAACCAT GAAGACAGGA TGGGGGCTGG CCTCTGGCTC TCATGGGGTC CAAGTTTTGT
3301 GTATTCTTCA ACCTCATTGA CAAGAACTGA AACCACCAAT ATGACTCTTG GCTTTTCTGT
3361 TTTCTGGGAA CCTCCAAATC CCCTGGCTCT GTCCCACTCC TGGCAGCAGT GCAGCAGGTC
3421 CAGGTCCGGG AAATGAGGGG TGGAGGGGGC TGGGCCCTAC GTGCTGTCTC ACACAGCCTG
3481 TCTGACCTCT CGACCTACCG GCCTAGGCCA CAAGCTCTGC CTACGCTGGT CAATAAGGTG
3541 TCTCCATTCA AGGCCTCACC GCAGTAAGGC AGCTGCCAAC CCTGCCCAGG GCAAGGCTGC
3601 AG

FIGURE 8C

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1 CCACCCCGGC CGCTCGCTGC GCTGCGCCGC ACCGCGCTGT CCTCCCGGAG CCGGACCGGG
61 GCCACCGCGC CCGCTCTGCT CCGACACCGC GCCCCCTGGA CAGCCGCCCT CTCCTCTAGG
121 CCCGTGGGGC TGGCCCTGCA CCGCCGAGCT TCCCGGGATG AGGGCCCCCG GTGTGGTCAC
181 CCGGCGCGCC CCAGGTCGCT GAGGGACCCC GGCCAGGCGC GGAG

Total number of bases is: 224.

FIGURE 9A

1 CCACCCCGGC CGCTCGCTGC GCTGCGCCGC ACCGCGCTGT CCTCCCGGAG CCGGACCGGG
61 GCCACCGCGC CCGCTCTGCT CCGACACCGC GCCCCCTGGA TCCCGGGATG AGGGCCCCCG
121 GTGTGGTCAC CCGGCGCGCC CCAGGTCGCT GAGGGACCCC GGCCAGGCGC GGAG

Total number of bases is: 174

FIGURE 9B

1 CCACCCCGGC CGCTCGCTGC GCTGCGCCGC ACCGCGCTGT CCTCCCGGAG CCGGACCGGG
61 GCCACCGCGC CCGCTCTGCT CCGACACCGC GCCCCCTGGA CAGCCGCCCT CTCCTCTAGG
121 CCCGTGGGGC TGGCCCTGCA CCGCCGAGCT GAGGGACCCC GGCCAGGCGC GGAG

Total number of bases is: 174

FIGURE 9C

1 CAGCCGCCCT CTCCTCTAGG CCCGTGGGGC TGGCCCTGCA CCGCCGAGCT TCCCGGGATG
61 AGGGCCCCCG GTGTGGTCAC CCGGCGCGCC CCAGGTCGCT GAGGGACCCC GGCCAGGCGC
121 GGAG

Total number of bases is: 124

FIGURE 9D

1 TCCCGGGATG AGGGCCCCCG GTGTGGTCAC CCGGCGCGCC CCAGGTCGCT GAGGGACCCC
61 GGCCAGGCGC GGAG

Total number of bases is: 74

FIGURE 9E

1 CCAGGTCGCT GAGGGACCCC GGCCAGGCGC GGAG

Total number of bases is: 34

FIGURE 9F

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1 CCAGGTGTGT CCACCTGGGC ATATCCACCA CCTCCCTCAC CAACATTGCT TGTGCCACAC
61 CCTCCCCCGC CACTCCTGAA CCCCCTCGAG GGGCTCTCAG CTCAGCGCCA GCCTGTCCCA
121 TGGACACTCC AGTGCCAGCA ATGACATCTC AGGGGCCAGA GGAAGTGTCC AGAGAGCAAC
181 TCTGAGATCT AAGGATGTCA

Total number of bases is: 200

FIGURE 10A

1 CCAGGTGTGT CCACCTGGGC ATATCCACCA CCTCCCTCAC CCACATTGCT TGTGCCACAC
61 CCTCCCCCGC CACTCCTGAA CCCCCTCGAG GGGCTCTCAG CTCAGCGCCA GCCTGTCCCA
121 TGGACACTCC AGTGCCAGCA ATGACATCTC

Total number of bases is: 150

FIGURE 10B

1 CCAGGTGTGT CCACCTGGGC ATATCCACCA CCTCCCTCAC CAACATTGCT TGTGCCACAC
61 CCTCCCCCGC CACTCCTGAA CCCCCTCGAG GGGCTCTCAG

Total number of bases is: 100

FIGURE 10C

1 CCAGGTGTGT CCACCTGGGC ATATCCACCA CCTCCCTCAC CAACATTGCT

Total number of bases is: 50

FIGURE 10D

1 CCAGGTGTGT CCACCTGGGC ATATCCACCC AGTGCCAGCA ATGACATCTC AGGGGCCAGA
61 GGAAGTGTCC AGAGAGCAAC TCTGAGATCT AAGGATGTCA

Total number of bases is: 100

FIGURE 10E

INTERNATIONAL SEARCH REPORT

Int. l. Application No

PCT/US 99/02258

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/505

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|---------------------------|
| X | WO 94 02611 A (NEW ENGLAND DEACONESS HOSPITAL) 3 February 1994 see claims 1,4-8,11-14,17-23 see figure 1; table 1 --- | 1-6,8-19 |
| X | WO 95 33057 A (MENARINI RICERCHE SUD SPA ;MELE ANTONIO (IT); SANTIS RITA DE (IT);) 7 December 1995 see claims 9,10,19,20 see examples 1-3 see figure 5; table 1 --- -/- | 1,2,4-6, 8,9,12, 13 |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- "E" earlier document but published on or after the international filing date
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- "&" document member of the same patent family

Date of the actual completion of the international search

24 June 1999

Date of mailing of the international search report

07/07/1999

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Fax: (+31-70) 340-3016

Authorized officer

van de Kamp, M

INTERNATIONAL SEARCH REPORT

Int. l. Application No

PCT/US 99/02258

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|---|-----------------------|
| X | HO ET AL.: "Use of a marked erythropoietin gene for investigation of its cis-acting elements" J. BIOL. CHEM., vol. 270, no. 17, 28 April 1995, pages 10084-10090, XP002105613 see abstract see figure 1 | 1-3,6,7, 10,11 |
| X | ----- MCGARY E C ET AL: "Post-transcriptional regulation of erythropoietin mRNA stability by erythropoietin mRNA-binding protein" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 13, 28 March 1997, pages 8628-8634, XP002084635 see the whole document | 1-3,6,7, 10,11 |
| X | ----- EP 0 409 113 A (BEHRINGWERKE AG) 23 January 1991 see claims 11,12 | 4,5,8,9, 12,13 |
| A | ----- BLANCHARD K L ET AL: "HYPOXIC INDUCTION OF THE HUMAN ERYTHROPOIETIN GENE: COOPERATION BETWEEN THE PROMOTER AND ENHANCER, EACH OF WHICH CONTAINS STEROID RECEPTOR RESPONSE ELEMENTS" MOLECULAR AND CELLULAR BIOLOGY, vol. 12, no. 12, 1 December 1992, pages 5373-5385, XP000562136 see the whole document | 1-3,6,7, 10,11 |
| P,X, L | ----- WO 99 02710 A (BETH ISRAEL HOSPITAL ;SYTKOWSKI ARTHUR J (US)) 21 January 1999 L: PRIORITY see claims 18-33 see example 7 ----- | 1-14,16 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 02258

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 5.9 and 13
are directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

See additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims: it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1 and 14 (both partially), 2-13 (all completely)

An isolated nucleic acid encoding erythropoietin wherein the nucleic acid has one or more mutations in a non-coding region, and wherein the erythropoietin has altered biological activity.

Erythropoietin encoded by these nucleic acids.

Uses of these erythropoietin molecules, e.g., as pharmaceuticals or in therapy.

2. Claims: 1 and 14 (both partially), 15 (completely)

An isolated nucleic acid encoding erythropoietin, comprising a mutation in the coding region encoding erythropoietin, the amino acid residue present in the corresponding position in wild-type erythropoietin being amino acid 101.

A mutant erythropoietin protein, wherein the amino acid residue at the position 101 is alanine.

Use of this erythropoietin mutant, e.g., as pharmaceutical or in therapy.

3. Claims: 1 and 14 (both partially), 16 (completely)

An isolated nucleic acid encoding erythropoietin, comprising a mutation in the coding region encoding erythropoietin, the amino acid residue present in the corresponding position in wild-type erythropoietin being amino acid 103.

A mutant erythropoietin protein, wherein the amino acid residue at the position 103 is selected from the group consisting of aspartate, alanine, glutamate, histidine and lysine.

Use of these erythropoietin mutants, e.g., as pharmaceuticals or in therapy.

4. Claims: 1 and 14 (both partially), 17 (completely)

As invention 2, but concerning amino acid 104.

5. Claims: 1 and 14 (both partially), 18 (completely)

As invention 2, but concerning amino acid 105.

6. Claims: 1 and 14 (both partially), 19 (completely)

FURTHER INFORMATION CONTINUED FROM PCT/SA/ 210

As invention 2, but concerning amino acid 108.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/02258

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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